Assessing the Flexibility of Intermediate Filaments by Atomic Force Microscopy

N. Mücke¹ ‡, L. Kreplak² *, R. Kirmse¹, T. Wedig³, H. Herrmann³, U. Aebi² and J. Langowski¹

¹Division Biophysics of Macromolecules
German Cancer Research Center, 69120 Heidelberg
Germany

²M.E. Müller Institute for Structural Biology
Biozentrum, University of Basel, Klingelbergstr. 70
4056 Basel, Switzerland

³Division Cell Biology
German Cancer Research Center, 69120 Heidelberg
Germany

Eukaryotic cells contain three cytoskeletal filament systems that exhibit very distinct assembly properties, supramolecular architectures, dynamic behaviour and mechanical properties. Microtubules and microfilaments are relatively stiff polar structures whose assembly is modulated by the state of hydrolysis of the bound nucleotide. In contrast, intermediate filaments (IFs) are more flexible apolar structures assembled from a ~45 nm long coiled-coil dimer as the elementary building block. The differences in flexibility that exist among the three filament systems have been described qualitatively by comparing electron micrographs of negatively stained dehydrated filaments and by directly measuring the persistence length of F-actin filaments (~3–10 μm) and microtubules (~1–8 mm) by various physical methods. However, quantitative data on the persistence length of IFs are still missing.

Toward this goal, we have carried out atomic force microscopy (AFM) in physiological buffer to characterise the morphology of individual vimentin IFs adsorbed to different solid supports. In addition, we compared these images with those obtained by transmission electron microscopy (TEM) of negatively stained dehydrated filaments. For each support, we could accurately measure the apparent persistence length of the filaments, yielding values ranging between 0.3 μm and 1 μm. Making simple assumptions concerning the adsorption mechanism, we could estimate the persistence length of an IF in a dilute solution to be ~1 μm, indicating that the lower measured values reflect constraints induced by the adsorption process of the filaments on the corresponding support.

Based on our knowledge of the structural organisation and mechanical properties of IFs, we reason that the lower persistence length of IFs compared to that of F-actin filaments is caused by the presence of flexible linker regions within the coiled-coil dimer and by postulating the occurrence of axial slipping between dimers within IFs.

Keywords: statistical analysis; persistence length; surface interaction; electron microscopy; atomic force microscopy

Introduction

One of the major functions of the cytoskeleton, a complex network of interconnected filaments (i.e. actin filaments, microtubules, and intermediate filaments), is to determine the shape and mechanical properties of cells. The physical properties of actin filaments and microtubules have been already well characterised at the single filament¹ ² and network levels.³ ⁴ In contrast, such knowledge is still sparse for intermediate filaments (IFs) and is urgently needed to understand how disease-causing inherited mutations in human IF proteins lead to impaired IF assembly and yield an increase in cell fragility in response to mechanical stress.⁵–⁷ All IF proteins exhibit a characteristic “tripartite” structure which includes an α-helical “rod”

*Corresponding author

Abbreviations used: IF, intermediate filament; AFM, atomic force microscopy; EM, electron microscopy; HOPG, highly oriented pyrolytic graphite.

E-mail address of the corresponding author:
laurent.kreplak@unibas.ch

‡ Correspondence concerning the software, e-mail: norbert.muecke@dkfz-heidelberg.de

© 2003 Elsevier Ltd. All rights reserved.
domain flanked by non-α-helical “head” and “tail” domains. The central rod domain contains a pronounced heptad repeat sequence of apolar residues exhibiting the signature of a coiled-coil structure. This periodicity is interrupted by three relatively short variable linkers L1, L12 and L2, leading to four independent α-helical coiled-coil segments, 1A, 1B, 2A and 2B. The elementary building block of all IF proteins is a dimer that can, under appropriate buffer conditions, self-assemble in vitro into 10 nm wide filaments. For cytoplasmic IFs, an intermediary stable complex is a tetramer comprising two dimers that are arranged in an antiparallel approximately half-staggered fashion relative to one another. In the case of vertebrate cytoplasmic IFs, the in vitro assembly process follows three distinct steps. First, the tetramers associate laterally into so-called “unit-length” filaments (ULFs). Second, the ULFs anneal longitudinally into filaments of variable width. Third, these filaments compact radially to form smooth-looking IFs 8–12 nm in diameter (Figure 1). The mechanical properties of IF networks in vitro have mainly been studied by rheological and light scattering methods. From these studies it was concluded that individual IFs are relatively flexible polymers and that IF networks exhibit “strain hardening” with a high resistance against rupture.

In order to obtain a more quantitative estimate of IF flexibility, human recombinant vimentin was assembled in vitro and the resulting IFs were adsorbed to various solid supports including mica, glass and HOPG (highly oriented pyrolitic graphite). Thus, immobilised filaments were imaged under physiological conditions by atomic force microscopy (AFM), and their contours were analysed using a statistical polymer chain approach. Depending on the support used, the apparent persistence length $\lambda_{app}$ of the vimentin IFs varied from 0.3 μm to 1 μm. The lower value was obtained with mica that interacts strongly with the filaments thereby yielding more compact structures, whereas the upper value was measured on glass that allows the filaments to adopt a contour that is energetically equilibrated to a higher extent in two dimensions.

**Theory**

The persistence length $\lambda$ of a filament is a measure of its flexibility and represents a statistical relationship between the contour length $s$ and the end-to-end distance $R$ of a given filament segment.

Let us consider a filament of contour length $s$, which is smoothly bent by an angle $\theta$ and energetically equilibrated in two dimensions. The energy necessary to maintain this configuration is given by:

$$E = Y I \theta^2 / 2s$$  \hspace{1cm} (1)

where $Y$ is the Young’s modulus, and $I$ the area moment of inertia of the filament.

The persistence length $\lambda$ of the filament can be directly related to these two quantities by:

$$Y I = k_B T \lambda$$  \hspace{1cm} (2)

where $k_B$ is the Boltzmann constant and $T$ is the absolute temperature.

Using equations (1) and (2), the normalised probability distribution function in two dimensions for a filament bent by an angle $\theta$ is Gaussian and can be written as:

$$P(\theta(s))_{2D} = \sqrt{\frac{\lambda}{2\pi s}} e^{-\lambda \theta^2 / 2s}$$  \hspace{1cm} (3)

Note that this distribution function depends on the contour length $s$ of the filament. In equation (3), the persistence length $\lambda$ appears as the characteristic length of the distribution. Hence for $\lambda \gg s$, the probability of bending the filament is equal to 0 (see also equation (4)).

From this distribution, it is possible to compute the mean-square angle and the normalised mean-fourth power of the angle:

$$(\theta^2(s))_{2D} = \frac{s}{\lambda}$$  \hspace{1cm} (4)

$$\frac{(\theta^2(s))_{2D}}{(\theta^4(s))_{2D}^{1/2}} = 3$$  \hspace{1cm} (5)

Using equation (3) the mean-square end-to-end distance of a filament of contour length $s$ can also be determined as:

$$\langle R^2(s) \rangle_{2D} = 4\lambda s \left( 1 - \frac{2\lambda}{s} \left( 1 - e^{-s/2\lambda} \right) \right)$$  \hspace{1cm} (6)
If a filament is energetically equilibrated on a solid support, its persistence length \( \lambda \) can be computed from two-dimensional images using equations (4) or (6). In that case and for contour lengths of the filaments in the range of \( \lambda \) the value obtained is identical to the persistence length of the same filament in a dilute solution. For contour lengths much larger than \( \lambda \) excluded volume effects may apparently stiffen the filaments. If the filament is not equilibrated, the above model only yields an apparent persistence length, which, in turn, depends on the surface adsorption mechanism.

**Results**

**Contour morphologies of vimentin IFs adsorbed to different solid supports**

A typical electron micrograph of negatively stained glutaraldehyde-fixed, vimentin filaments exhibits smooth-looking structures approximately 10 nm in diameter (Figure 1).

For comparison, Figure 2 displays tapping-mode AFM images in air (Figure 2a) or in physiological buffer (Figure 2b) of vimentin IFs adsorbed to mica, to HOPG (Figure 2c) and to hydrophilic glass (Figure 2d). On all these supports, the filaments appear to be stably attached without the need of any chemical cross-linking. Filaments on mica imaged in air show two different morphologies (Figure 2a) characterised by a height of 0.8 nm and 1.7 nm, respectively, and a full width at half maximum (FWHM) of 100 nm and 45 nm, respectively. The first type may correspond to filaments that have been spread flattened into protofilaments, whereas the second type may correspond to smooth-looking dried filaments. Notice that in our AFM images no individual protofilamentous substructures could be depicted, contrary to previously described electron micrographs of unravelled filaments.18

In contrast, when imaged in buffer, the filaments adsorbed to the three different supports appear compact and smooth with a height of 3–5 nm and an apparent diameter of 40–70 nm. For comparison, neurofilaments exhibit a height of 9.5 nm and a FWHM of 50 nm when covalently bound to a modified glass substrate and imaged by contact mode AFM in buffer.19 However, even if the filaments have a similar height and apparent diameter on the three different supports, they yield very different contours. On mica (Figure 2b), for example, the filaments often show loops in comparison to a more extended appearance of the filaments when adsorbed to HOPG (Figure 2c), hydrophilic glass (Figure 2d), or to carbon-coated copper grids (Figure 2e). Such differences in contour are a first hint that the vimentin IFs exhibit somewhat different flexibilities when adsorbed to either mica or to the other three supports.

**Statistical analysis of IF contours with a 2D equilibration model**

In order to quantify the apparent flexibility of vimentin IFs after adsorption to the different supports, we used the AFM images to measure the mean-square end-to-end distance \( \langle R^2 \rangle_{\text{app}} \) of entire filaments with contour lengths ranging from 2000 nm to 4000 nm (see Table 1). For each filament set, a mean contour length \( \langle s \rangle \) was also computed and used in equation (6) to estimate an apparent persistence length \( \lambda_{\text{app}}^2 \) (Table 1). As expected from the contour differences observed on the AFM and EM images, the vimentin IFs adsorbed to mica have a lower apparent persistence length (337 nm) than the same filaments adsorbed to HOPG (675 nm), carbon-coated copper grids (967 nm), or hydrophilic glass (1061 nm).

In order to confirm these results and to verify the validity of our statistical model, the angle distribution function \( \Theta \) was estimated as a function of the measured contour length \( s \). For this purpose, the filament contours were divided into sets of segments of fixed contour length \( s \). For each support and a fixed contour length \( s \) of 1000 nm, an example of a \( \Theta \) distribution function is displayed in Figure 3a. In each case the distributions could be fitted with similar Gaussian functions, except for mica where the distribution was significantly broader. As stressed when introducing equation (3), the statistical analysis that we have chosen is only valid if the \( \Theta \) distribution function is Gaussian. This property was checked by plotting the normalised mean-fourth power of the angle distribution \( (\Theta^4)/(\Theta^2)^2 \) as a function of the contour...
Table 1. Polymer statistics of IFs adsorbed to different supports

<table>
<thead>
<tr>
<th>Surface</th>
<th>$(\Theta^4)/(\Theta^2)^2$</th>
<th>$\lambda_{(\Theta^2)}^2$ (nm)</th>
<th>$(R^2)_{\text{app}}$ (nm$^2$)</th>
<th>$\langle s \rangle$ (nm)</th>
<th>$\lambda_{(\Theta^4)}^2$ (nm)</th>
<th>Counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>EM grid</td>
<td>3.08 ± 0.13</td>
<td>847</td>
<td>$3.60 \times 10^6$</td>
<td>2266 ± 409</td>
<td>967</td>
<td>51</td>
</tr>
<tr>
<td>HOPG</td>
<td>2.30 ± 0.18</td>
<td>769</td>
<td>$3.97 \times 10^6$</td>
<td>2626 ± 669</td>
<td>675</td>
<td>42</td>
</tr>
<tr>
<td>Mica</td>
<td>2.73 ± 0.09</td>
<td>284 (251*)</td>
<td>$2.54 \times 10^6$</td>
<td>2545 ± 495</td>
<td>337</td>
<td>57</td>
</tr>
<tr>
<td>Glass</td>
<td>3.02 ± 0.09</td>
<td>980</td>
<td>$4.37 \times 10^6$</td>
<td>2497 ± 458</td>
<td>1061</td>
<td>173</td>
</tr>
</tbody>
</table>

For each support, we have computed the apparent mean-square end-to-end distance $(R^2)_{\text{app}}$, the mean contour length $\langle s \rangle$, and the normalised mean-fourth power of the angle distribution $(\Theta^4)/(\Theta^2)^2$. The persistence lengths $\lambda_{(\Theta^2)}$ and $\lambda_{(\Theta^4)}$ were estimated from the experimental end-to-end distances and angle distributions, respectively, using the 2D equilibration model (equations (4)–(6)) described in Theory. All filaments had contour lengths ranging from 2000 nm to 4000 nm.

* Linear fit with a non-zero constant, dotted line in Figure 3c.

Figure 3. Statistical analysis of filament contours for vimentin IFs adsorbed to hydrophilic glass (black), HOPG (blue), mica (green) and to electron microscopy grids (red). All the filaments had a contour length in the range of 2000 nm to 4000 nm. a, Angle distribution function $\Theta$ for $s = 1000$ nm. Continuous lines represent a Gaussian fit of the experimental data. b, Normalised mean-fourth power of the angle distribution $(\Theta^4)/(\Theta^2)^2$ as a function of contour length. A value of 3 (straight line) corresponds to a Gaussian distribution function. c, Plot of the mean-square angle $(\Theta^2)$ as a function of contour length. Each data set is fitted with equation (4) (continuous line) for contour length in the range 600–1600 nm. For mica, equation (4) does not fit properly and we also show, as a comparison, the result of a linear fit with a non-zero constant (broken line). d, Plot of the mean-square end-to-end distance as a function of the contour length.
length $s$ (Figure 3b). For contour lengths shorter than 600 nm, some deviation from the theoretical value of 3 (see equation (5)) is observed due to the finite pixel size and the smoothing procedure. Above 600 nm, it was possible to estimate the apparent persistence length $\lambda_{\text{app}}$ for each support by fitting the behaviour of the mean-square angle $\langle \theta^2 \rangle$ as a function of contour length (Figure 3c) with equation (4). The measured apparent persistence lengths $\lambda_{\text{app}}$ (see Table 1) are 284 nm (mica), 769 nm (HOPG), 847 nm (carbon-coated copper grid), and 980 nm (glass), respectively. These values are consistent with the corresponding $\lambda_{\text{app}}$ values (see Table 1 and above).

However, it is interesting to note that the linear fits shown in Figure 3c were accurate for the glass and the EM data sets but rather poor for the mica data set and at high contour lengths for the HOPG data set. This clearly indicates that for the first two data sets, the vimentin IFs can effectively be modelled as smoothly bent strings equilibrated in two dimensions. In other words, the influence of the glass and the carbon-coated copper grid on the vimentin IF flexibility is negligible and consequently the apparent persistence length obtained for these two supports, $\sim 1000$ nm, should be similar to the persistence length of an unconstrained single vimentin IF in a dilute solution.\(^{17,20}\)

In contrast, the filaments adsorbed to mica and HOPG appear more flexible, as indicated by their lower apparent persistence length. For these two supports, a modified version of our statistical model has to be used to analyse the data, i.e. one that takes into account some kind of filament-support interaction.

### The contours of the IFs adsorbed to mica can be described by a quasi-normal projection model

Since it is difficult to know the type of interaction that drives the adsorption of vimentin IFs to mica or HOPG, we have decided to use a practical first approximation: if the filament-support interaction is strong enough, a vimentin filament may adsorb to the support as an apparent normal projection of the three-dimensional filament contour onto the plane. Note that the term projection is geometrically incorrect here, since the actual contour length of the filament is conserved upon adsorption. However, by such an approximation the mean-square of the projected end-to-end distance becomes:\(^{17}\)

$$\langle R^2 \rangle_{\text{proj}} = \langle R^2 \rangle_2 + \langle R^2 \rangle_3 = \frac{2}{3} \langle R^2 \rangle_{3D}$$

(7)

For $s \to \infty$:

$$\langle R^2 \rangle_{\text{proj}} = \frac{1}{3} \langle R^2 \rangle_{2D}$$

(8)

where $\langle R^2 \rangle_{3D}$ is the mean-square end-to-end distance of the filament equilibrated in a dilute solution, and $\langle R^2 \rangle_{2D}$ is the mean-square end-to-end distance of the filament equilibrated on a solid support (see equation (6)). $\langle R^2 \rangle_{3D}$ and the persistence length $\lambda$ of the filament equilibrated in a dilute solution is given by:\(^{14}\)

$$\langle R^2 \rangle_{3D} = 2\lambda s \left( 1 - \frac{\lambda}{s} \left( 1 - e^{-s/\lambda} \right) \right)$$

(9)

To test the validity of the above model, the experimental mean-square end-to-end distance of filaments, with contour lengths ranging between 2000 nm and 4000 nm, was calculated as a function of their contour length $s$. The corresponding plots are shown in Figure 3d for each support. For $s$ longer than 600 nm, the $\langle R^2 \rangle$ of the filaments adsorbed to mica is smaller and deviates strongly from the $\langle R^2 \rangle$ of the filaments adsorbed to the three other supports. If we assume that the mean-square end-to-end distances measured for the filaments adsorbed to glass or EM grids are a good approximation of $\langle R^2 \rangle_{2D}$, the filaments adsorbed to mica are most likely to follow equation (8). As the extrapolation to an infinite contour length of our experimental data is not very reliable, we decided to use another test of the quasi-normal projection model.

According to equations (7) and (9), it should be possible to estimate the “true” persistence length of the vimentin IFs equilibrated in a dilute solution from the experimental mean-square end-to-end distance of the filaments adsorbed to mica. The value obtained for $\lambda$ should be similar to that obtained from the glass data set (i.e. $\sim 1000$ nm), assuming that the filaments are energetically equilibrated on this support (2D equilibration hypothesis). For this purpose, we determined the persistence length as a function of contour length using the experimental mean-square end-to-end distances of the filaments adsorbed to glass and to mica (Figure 4). For this test, the filaments analysed had contour lengths in the range of 3000 nm to 5500 nm.

When the 2D equilibration hypothesis (equation (6)) is used for the mica and glass data sets (Figure 4, diamonds), both curves show an increase of $\lambda$ at low contour length due to the finite pixel size and the smoothing procedure. At high contour length, $\lambda$ is constant and the values obtained for the two data sets are similar to those obtained previously and listed in Table 2, 358 nm for mica and 1107 nm for hydrophilic glass.

When the quasi-normal projection hypothesis (equations (7) and (9)) is used for the mica data set (Figure 4, squares), $\lambda_{\text{app}}$ is equal to 1275 nm at high contour length (see Table 2), which is only 15% higher than the value $\lambda_{\text{app}}$ obtained for the glass data set using the 2D equilibration model (1107 nm). However, for filaments with contour lengths below 2500 nm, $\lambda$ is increasing steadily, indicating that the adsorbed filaments cannot be considered as quasi-normal projections any more.

In conclusion, for filaments longer than 2500 nm adsorbed to mica, it is possible to calculate with...
(equation (6)) was used to estimate the persistence length \( l \). The quasi-normal projection model was used to estimate the filaments adsorbed to each support. For mica only, the interaction energy in the range of the thermal energy, the filaments will freely equilibrate on the support before they become adsorbed. In this case, the elastic properties are conserved during the adsorption process, so that the measured persistence length is equal to that of a filament equilibrated in a dilute solution.

1. For an interaction energy in the range of the thermal energy, the filaments will be “caught” by the support before having equilibrated so that the filaments are “fixed” into a contour resembling a normal (i.e. perpendicular) projection of the actual three-dimensional contour onto the support. Such a “capture” mechanism yields more condensed filaments on the support and hence a smaller apparent persistence length is revealed.

Vimentin IFs readily adsorb to a wide range of solid supports, with very different surface properties, either being highly negatively charged (mica), hydrophobic (HOPG) or polar (hydrophilic glass). However, on each support the filaments exhibit different flexibilities as emphasised by the broad range of measured apparent persistence lengths (see Figure 2 and Table 1). Similar effects are obtained by varying the adsorption buffer with a given support (data not shown). Such kind of buffer-dependences of the apparent flexibility has been extensively studied by AFM for DNA molecules adsorbed to mica and is caused by different kinds of mica–DNA interaction. To our knowledge, this is the first time that a similar effect has been reported with protein filaments. Strikingly, although DNA and vimentin IFs are very different polymers, upon adsorption to a solid support their contours can be accurately described by the linear chain statistics model presented in Theory. With both polymers, depending on the filament-support interaction employed, two principal adsorption scenarios are possible.

The apparent flexibility of vimentin IFs depends on the type of support

<table>
<thead>
<tr>
<th>Surface</th>
<th>Mica</th>
<th>Glass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Counts (nm)</td>
<td>57</td>
<td>56</td>
</tr>
<tr>
<td>( (\sigma) ) (nm)</td>
<td>3912 ± 592</td>
<td>3501 ± 649</td>
</tr>
<tr>
<td>( (R^2)_{app} ) (nm(^2))</td>
<td>4.58 \times 10^6</td>
<td>7.72 \times 10^6</td>
</tr>
<tr>
<td>( (R^2)_{2D} ) (nm(^2))</td>
<td>6.87 \times 10^6</td>
<td>1107</td>
</tr>
<tr>
<td>( \lambda_{app} ) (nm)</td>
<td>358</td>
<td>1275</td>
</tr>
<tr>
<td>( \lambda_{3D} ) (nm)</td>
<td>106</td>
<td>7.72</td>
</tr>
</tbody>
</table>

Table 2. Comparison of the two adsorption models

For mica and glass, we have used filaments with contour lengths ranging from 3000 nm to 5500 nm to compute the apparent mean-square end-to-end distance \( (R^2)_{app} \) and the mean contour length \( (\sigma) \). As in Table 1 the 2D equilibration model (equation (6)) was used to estimate the persistence length \( \lambda_{app} \) of the filaments adsorbed to each support. For mica only, the quasi-normal projection model was used to estimate \( (R^2)_{3D} \) from \( (R^2)_{app} \) (equation (7)). In turn, \( (R^2)_{3D} \) was used in combination with \( (\sigma) \) in equation (9) to estimate \( \lambda_{3D} \).

Vimentin IFs equilibrate upon adsorption to glass or to a carbon film (i.e. a glow-discharged carbon-coated EM grid). In contrast, the same filaments appear normally projected when adsorbed to mica. After proper modelling, both data sets yielded a similar estimate of the persistence length \( \lambda \), i.e. 1000–1300 nm. Strikingly, a similar estimate has been recently derived by Fudge et al. using the
initial tensile modulus of hagfish slime threads containing mainly keratin-like IFs.\textsuperscript{21}

Concerning the high binding affinity of IFs to different solid supports, it is well known that the nuclear lamins interact with the nuclear surface of the nuclear envelope.\textsuperscript{22} Similarly, vimentin does associate \textit{in vivo} with the plasma membrane.\textsuperscript{23} The lipids composing the plasma membrane have generally polar head groups that may interact with the vimentin IFs in a way similar to the Si–OH groups of the hydrophilic glass. Hence independent of any interaction mediated by membrane proteins, vimentin IFs may \textit{in vivo} strongly bind to and equilibrate on the plasma membrane, thereby forming a mechanically stable moiety similar to that observed in the eye lens.\textsuperscript{23}

\textbf{Molecular origin of IF flexibility}

One would assume that in the case of protein filaments such as, for example, vimentin IFs, a physical parameter like the persistence length should be directly related to their supramolecular architecture and the interactions between neighbouring subunits within the filament. The average vimentin IF cross-section typically contains 16 coiled-coil dimers (~45 nm long each) that are assumed to be aligned approximately parallel to the filament axis. Depending on the position in the filament, adjacent dimers can be oriented parallel or antiparallel, un staggered or approximately half-staggered. The lateral interactions between dimers appear relatively strong, since IFs are able to withstand the combined challenge of the Si–OH groups of the hydrophilic glass. Hence independent of any interaction mediated by membrane proteins, vimentin IFs may \textit{in vivo} strongly bind to and equilibrate on the plasma membrane, thereby forming a mechanically stable moiety similar to that observed in the eye lens.\textsuperscript{23}

Comparing the flexibility of IFs and F-actin filaments

The basic principles that we used to describe qualitatively the flexibility of IFs should be also valid for other protein filaments such as F-actin filaments and microtubules. The case of F-actin filaments is particularly interesting. Depending on buffer conditions and the presence of binding proteins, they exhibit a persistence length ranging between 3 \textmu m and 10 \textmu m,\textsuperscript{32,33} which is at least threefold larger than the values measured for vimentin IFs. IFs and F-actin filaments can reach similar lengths and they reveal a similar outer diameter, i.e. 10 nm and 9 nm, respectively. However, the molecular architecture of the two filaments is very different, typically yielding a mass-per-length of ~16 kDa/nm for F-actin filaments\textsuperscript{34} and ~36 kDa/nm for vimentin IFs.\textsuperscript{35} Intuitively, since both filaments are protein polymers, we would predict that the filament with the lower mass-per-length, i.e. the F-actin filament, should be more flexible than the vimentin IF. This apparent contradiction may be best explained by analysing the molecular origin of the bending stiffness of F-actin filaments. To a first approximation, F-actin is a two-stranded helical filament built from a 43 kDa globular subunit. The inter-subunit interactions along the two long-pitch helical strands are generally considered stronger than those occurring between the two strands.\textsuperscript{36–38} A \textit{priori} this particular inter-subunit bonding pattern allows for some lateral slipping between adjacent subunits of the two strands.\textsuperscript{39} Practically, as the registration between the two strands is fairly well defined, only a small amount of axial slippage occurs. In other words, the two strands forming
the F-actin filament are tightly bound together yielding a higher persistence length than the one measured for vimentin IFs.

As for IFs, the amount of axial molecular motion that can take place without disrupting the molecular architecture appears to be the key factor governing the flexibility of F-actin filaments. Following this basic principle, the effect of cations, drugs or interacting proteins on the flexibility of IFs and F-actin filaments could be readily understood.43

**Biological significance of IF flexibility?**

Not only do actin filaments and microtubules serve as “tracks” for molecular motors to move cargoes, but they are also responsible for the dynamic properties of the cytoskeleton which, in turn, determine cell plasticity and drive cell motility and remodelling of cell shape. In contrast, the cytoplasmic IF network is generally considered as a relatively inert and mechanically resistant scaffold.40 This picture appears somehow contradictory, since IFs are more flexible than F-actin filaments and microtubules, as elaborated here. The molecular origin of this flexibility, i.e. a high potential for axial sliding of the dimers relative to one another within the filament, may qualify cytoplasmic IFs as mechanical signal transducers.41

In this context, cells and tissues exhibit a wide spectrum of responses to mechanical stimuli like, for example, shear stress. This indicates that a mechanical signal may be transduced, i.e. via the IF network, from the plasma membrane to the nucleus where a specific response is triggered.42 43

Based on immunofluorescence studies of epithelial cells, keratin IF bundles, i.e. tonofibrils, have been implicated in the transmission of mechanical forces throughout the cytoplasm.44 45 These tonofibrils also reveal wave-like patterns or kinks that can propagate along their length in synchrony with phosphorylation/dephosphorylation cycles.46 This spatial propagation of a distinct filament deformation pattern along the length of an IF bundle is in agreement with our in vitro model of vimentin IF flexibility involving an axial sliding of dimers relative to one another. Ideally this sliding process can proceed all along the length of the filament in a very effective manner, i.e. without disrupting its molecular architecture.

In contrast, the rigidity and inter-subunit coupling of F-actin filaments is such that a local deformation is only propagated over a few subunits before it is “fading out” or even reversed.39

**Materials and Methods**

**Recombinant vimentin**

Human vimentin was expressed and purified as described.46 The protein was stored at −80 °C in 8 M urea, 5 mM Tris–HCl (pH 7.5), 1 mM DTT, 1 mM EDTA, 0.1 mM EGTA, 10 mM methyl ammonium chloride. The day before use, the protein was dialysed into 2 mM sodium phosphate (pH 7.5), 1 mM DTT, at room temperature by lowering the urea concentration in a step-wise fashion (6 M, 4 M, 2 M, 0 M). Dialysis was continued overnight at 4 °C into fresh buffer without urea. The next day the protein was dialysed for one hour at 4 °C into 2 mM sodium phosphate (pH 7.5). Filament assembly was performed by adding an equal volume of 200 mM KCl in 2 mM sodium phosphate (pH 7.5) to a 0.4 mg/ml vimentin solution, at 37 °C. After 20 minutes to one hour, the filament solution was diluted 1:80 to 1:300 in 2 mM sodium phosphate (pH 7.5), 100 mM KCl, and 30 μl aliquots were allowed to adsorb to a solid support for at least five minutes prior to AFM imaging.

**Surface preparation**

Three kinds of solid supports were used in this study: Muscovite mica, highly oriented pyrolytic graphite (HOPG), and hydrophilic glass. Mica and HOPG were freshly cleaved immediately before use. To obtain a hydrophilic glass surface, we used the following protocol: Standard light microscopy cover slips were washed with ethanol and water, and then exposed for one hour to “Piranha solution”, a mixture of three parts H2SO4 (97%) and one part H2O2 (30%). Next, the cover slips were rinsed in water, exposed twice to ultrasound waves (50 kHz) for ten minutes, and finally dried under a stream of nitrogen. After such treatment, the surface is covered by Si–OH groups that are stable for a few hours.

The supports were mounted for AFM imaging in two different ways, both of which proved equally suited. Either, a small piece of support was glued onto a Teflon disc by water-insoluble epoxy glue (Araldit; Ciba-Geigy, Basel, Switzerland). The Teflon disc was then glued (cyanoacrylate glue) to a steel disc and mounted onto the piezoelectric scanner. Or alternatively, a large piece of support was directly glued to a steel disc.

**Electron microscopy**

For electron microscopy (EM), filament assembly was terminated after 20 minutes to one hour by the addition of an equal volume of stop buffer (0.2% (w/v) glutaraldehyde in 100 mM KCl, 2 mM sodium phosphate, pH 7.5). After three to five minutes, 5 μl aliquots were adsorbed for one minute to glow-discharged carbon-coated copper grids and negatively stained with 2% (w/v) uranyl acetate for visualisation using a Zeiss 900 transmission electron microscope (Carl Zeiss, Oberko-chen, Germany). For image processing, prints enlarged five times from the original negative (50,000 ×) were digitised.

**Atomic force microscopy**

For scanning in air the sample was prepared by the following protocol: 20 μl of assembled vimentin IFs diluted to 0.5–2 μg/ml were adsorbed for ten minutes to a freshly cleaved piece of mica. The mica was then washed carefully with 300 μl of assembly buffer (2 mM sodium phosphate (pH 7.5), 100 mM KCl) to remove all non-bound filaments. Next, the filaments attached to the mica were incubated for ten minutes with 100 μl glutaraldehyde (0.5% in 2 mM sodium phosphate, pH 7.5) to stabilise their structure before they were washed with...
2 ml distilled water and dried under a steady stream of nitrogen.

For operating in air we used 125 μm long silicon cantilevers (type NCH from Nanosensors, Neuchâtel, Switzerland), which had a nominal spring constant of 21–78 N/m. The cantilever drive frequency was chosen between 200 kHz and 300 kHz, and the scanner drive frequency was fixed to 3 Hz.

For scanning in liquid the sample was prepared by adsorbing 20 μl of assembled vimentin IFs, diluted to 0.5–2 μg/ml, for five minutes to the different solid supports described above.

We used 100 μm long cantilevers with oxide sharpened silicon nitride tips, which had a nominal spring constant of 0.38 N/m (type NP-S from Digital Instruments, Santa Barbara, USA). The cantilever drive frequency was chosen between 7.5 kHz and 9.5 kHz, and the scanner drive frequency was fixed to 2 Hz.

All AFM images were recorded in tapping-mode using a Nanoscope IIIa running with software version 5.12r3 (Digital instruments, Santa Barbara, USA), operated at room temperature. 512 × 512 pixels images were recorded at a scan size of either 10 μm or 20 μm, thus resulting in pixel sizes of 20 nm or 40 nm, respectively. For further image processing, 10 μm size zooms were extracted from the 20 μm scans using the Nanoscope III software.

AFM images were processed using the ImageJ software. ImageJ is a version of the NIH-Image software developed by the National Institutes of Health. It is a public domain software†. Filament contours were traced and exported as XY-coordinate sets by using either the “Freehand Linetool” or a skeleton algorithm implemented in Image J.

Data analysis

The XY-coordinate sets of the filament contours were analysed using Thetascan 1.0 developed by N. Mücke in Origin 7.0. To assess the influence of pixel number and the errors introduced by the freehand tracing of the filament contours, computer-generated filaments were first pixelised and their contours traced in the same way as done with the experimental data. It was found that the experimental data acquisition errors can be minimised by a smoothing procedure using the weight average of five contiguous XY-coordinates centred about a given XY-coordinate:

\[
V_{i,\text{correct}} = \frac{1V_{i-2} + 2V_{i-1} + 4V_i + 2V_{i+1} + 1V_{i+2}}{10}
\]

where \(V_i\) is the vector of the tangent to the curve on XY-coordinate \(i\). Note that this procedure removes two points at each end of the filament. Next, the corrected XY-coordinate sets of the filament contours were split into segments of increasing contour length in 100 nm increments. For each segment set of contour length \(s\), the mean-square angle \((\Theta^2)^i/(\Theta^2)^R\) and the mean-square end-to-end distance \((R^2)\) were computed.

Acknowledgements

We thank S. Stoll for discussions and support concerning the data analysis software, and J. Spatz for discussions concerning the glass modification protocol. L.K. was supported by a fellowship awarded by the “Fondation pour la Recherche Médicale”. The work was funded by a grant awarded to H.H. by Deutsche Forschungsgemeinschaft (DFG, HE1853/4-1), an NCCR program grant on “Nanoscale Science” awarded to U.A. by the Swiss National Science Foundation, The M.E. Müller Foundation of Switzerland, and the Canton Basel Stadt.

References


†http://rsb.info.nih.gov/ij

Edited by M. Moody

(Received 30 July 2003; received in revised form 19 November 2003; accepted 19 November 2003)