A Quantitative Kinetic Model for the in Vitro Assembly of Intermediate Filaments from Tetrameric Vimentin*

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In vitro assembly of intermediate filament proteins is a very rapid process. It starts without significant delay by lateral association of tetramer complexes into unit-length filaments (ULFs) after raising the ionic strength from low salt to physiological conditions (100 mM KCl). We employed electron and scanning force microscopy complemented by mathematical modeling to investigate the kinetics of in vitro assembly of human recombinant vimentin. From the average length distributions of the resulting filaments measured at increasing assembly times we simulated filament assembly and estimated specific reaction rate parameters. We modeled eight different potential pathways for vimentin filament elongation. Comparing the numerical with the experimental data we conclude that a two-step mechanism involving rapid formation of ULFs followed by ULF and filament annealing is the most robust scenario for vimentin assembly. These findings agree with the first two steps of the previously proposed three-step assembly model (Herrmann, H., and Aebl, U. (1998) Curr. Opin. Struct. Biol. 8, 177–185). In particular, our modeling clearly demonstrates that end-to-end annealing of ULFs and filaments is obligatory for forming long filaments, whereas tetramer addition to filament ends does not contribute significantly to filament elongation.

Intermediate filaments (IFs) represent one of the three major cytoskeletal filaments of metazoan cells. Together with microfilaments (MFs) and microtubules (MTs), they form the cytoskeleton of the cell, which participates in many cellular functions, including cell division and plasticity, mechanical stress resistance, and organelle transport. Whereas the basic building blocks of MFs and MTs, i.e. actin and tubulin, are globular proteins, those of IFs are elongated with a central α-helical rod domain such that two molecules associate in a parallel, unstaggered fashion to form an extended coiled-coil dimer. In addition, whereas actins and tubulins are ATPases and GTPases, respectively, IF proteins do not have any known enzymatic activity (1). The self-assembly process of MFs and MTs, whose kinetics have been modeled a long time ago, proceeds via polar association of globular subunits into filamentous structures (2–5). In contrast, the assembly mechanism of IFs is far less understood. However, for a more rational understanding of the self-organization of cellular supramolecular structures, kinetic modeling of their assembly mechanism is a prerequisite (2, 3, 5). Moreover, the fact that MFs and MTs are highly dynamic structures with subunits constantly being removed from one end while being added to their other end, represents a direct consequence of their polar structural nature, i.e. functionally harboring a “fast growing” and a “slow growing” end (6–8). In contrast, IFs are supposed to be non-polar and their assembly pathway is rather different involving lateral and longitudinal non-polar growth (9).

In vertebrates, the multigene family of IF proteins consists of more than 65 members (10), which have been grouped with respect to their primary amino acid sequence identity into five different sequence homology classes. Keratins belong to the type I and II sequence homology classes, form obligate heterodimeric complexes and are expressed in epithelia (11). IF proteins from sequence homology classes III and IV such as vimentin, desmin, neurofilaments, and nestin, are expressed in mesenchymal, muscular, and neuronal tissue, respectively (12). Type III and IV IF proteins are able to coassemble with one another, but not with type I and II proteins. In addition to cytoplasmic IF proteins, vertebrates harbor three nuclear IF genes leading to the differential expression of four major proteins, i.e. lamin A/C, B1, and B2, which exhibit specific features reflecting their distinct nuclear functions (1, 13). These type V IF proteins assemble neither with keratins nor vimentin-type molecules (1, 11).

IFs are highly insoluble in physiological buffer and can be solubilized only after denaturation with a chaotropic agent such as, for example, 8 M urea. The first stable soluble oligomers found in vitro after reconstitution in a buffer without urea are tetramers made from two coiled-coil dimers, which are associ-
ated approximately half-staggered and in an antiparallel fashion (14). After initiation of assembly by rapid dilution into physiological ionic strength buffer, tetramers interact laterally without any lag to yield so-called unit-length-filaments (ULFs), which are ~60 nm long and 17 nm thick, and exhibit a mass-per-length identical to mature IFs (15, 16). As documented by time-lapse electron microscopy, ULFs subsequently anneal longitudinally, first into immature filaments, which then radially compact to yield mature IFs (15). In the case of certain desmin disease mutants, assembly stops at the ULF stage, indicating that ULFs are bona fide IF assembly intermediates (14, 18).

We now have investigated for the first time the IF self-assembly mechanism quantitatively by using mathematical modeling of the kinetic reaction parameters. For this purpose we followed the assembly process of recombinant vimentin on a time scale of seconds by determining the length distribution of growing filaments at distinct time intervals by electron microscopy (EM) and scanning force microscopy (SFM) in vitro. In parallel, the time-dependent mean filament length (MFL) was calculated and integrated into a set of models, which describes the IF assembly dynamics. The models were then tested in terms of the previously proposed three-phase IF assembly model suggesting (i) formation of ULFs within seconds; (ii) massive longitudinal annealing of the ULFs into long filaments correlating with a steep increase in viscosity of the assembly solution between 1 and 5 min (3, 15); and (iii) radial compaction of the loosely packed filaments into mature IFs, starting at ~5 min (15, 16). In testing this three-phase IF assembly model, we assumed different modes of filament elongation, including tetramer association, ULF addition, and longitudinal annealing of filaments. Considering all possible combinations, we came up with eight different scenarios for the IF assembly process. By fitting these to the time-dependent measured filament length data, we were able to define the most likely assembly scenario. Moreover, this approach allowed us to estimate the essential kinetic parameters of the assembly reaction. The most likely assembly scenario was validated by subjecting it to a sensitivity analysis with respect to its kinetic parameters and assessing the influence of the different reaction steps on the overall assembly scheme.

**EXPERIMENTAL PROCEDURES**

**Vimentin Preparation and Assembly**

Recombinant human wild-type vimentin was produced and purified from *Escherichia coli*, strain TG1 (15). Vimentin was dialyzed from 8 mM urea in a stepwise fashion (6, 4, and 2 mM urea) into 2 mM sodium phosphate buffer at pH 7.5 ("P, buffer") as described previously (19, 20). In vitro assembly was started by adding KCl to a final concentration of 100 mM at 37 °C. For SFM, the filaments were diluted at different time points immediately diluted 1:80 in P, buffer containing 100 mM KCl ("assembly buffer"), immediately before a 30-μl aliquot was deposited on a solid support (see below). After 2 min of adsorption, samples were washed two times with 100 μl of assembly buffer each and imaged. For EM, filament assembly was stopped by the addition of an equal volume of assembly buffer containing 0.2% (w/v) glutaraldehyde. 5-μl sample drops were then placed on carbon-coated copper grids.

**SFM**

Images were recorded at room temperature in buffer using the fluid cell of a MultiMode™ microscope with a Nanoscope IIIa controller running the software version 5.12r3 (Veeco, Santa Barbara, CA). Two types of solid supports were used in SFM: freshly cleaved mica and hydrophilic glass. The solid supports were prepared as described in previous studies (19, 20). We used 100-μm-long cantilevers with oxide-sharpened silicon nitride tips having a nominal spring constant of 0.38 N/m (type NP-S from Veeco). All images were recorded in tapping mode as 512 × 512-pixel frames covering 8 × 8-μm sample areas using a scanning speed of 1.5 Hz and a cantilever drive frequency ranging between 7.5 and 9.5 kHz. Images were processed using the Nanoscope software and ImageJ (21).

**Electron Microscopy**

All images were recorded with a Zeiss 900 transmission electron microscope (Carl Zeiss, Oberkochen, Germany) on Kodak SO 163 image plates. For specimen support, carbon-coated EM grids were used that were prepared as follows: thin carbon films were produced in a Baltec SCD 005 sputter coater equipped with a CEA 035 carbon evaporation supply. After evacuation, the chamber was flushed seven times with nitrogen to further remove oxygen. The carbon films, which were evaporated onto mica, were next floated onto a water surface from where they were picked up by plain EM grids (3.05-mm diameter copper grids, 300 square meshes per inch, Agar Scientific). Prior to use, the EM grids were rendered hydrophilic by 2-min glow discharge in a reduced atmosphere of air in the Baltec SCD 005 device.

After adsorption of 5-μl aliquots onto the EM grids for 2 min, the samples were washed with distilled water and negatively stained using 2% uranyl acetate (w/v). For image processing the EM film plates were digitized with an Epson Perfection 3170 Photo scanner at 800 dpi and saved as TIF files. ImageJ (21) was used to measure the contour length of the filaments.

**Kinetic Models**

A qualitative description for the assembly of IFs suggests three distinct major phases: (i) ULF formation; (ii) longitudinal annealing of ULFs and growing filaments; and (iii) radial compaction of immature filaments into mature IFs (16). On this basis we described the assembly as a sequence of successive bimolecular reactions that result in the formation of ULFs and elongated filaments. Similar approaches for modeling protein self-assembly have been described by Oosawa and co-workers (22). Note that here we have not modeled the compaction event.

Knowing the number of tetramers per μm of filament (16), the so-called linear density, we can express the MFL at any given time. On average, eight tetramers constitute one ULF, and 23 ULFs are contained within 1 μm of filament equivalent to 184 tetramers (13). The models used are as follows.
"Simple" Model: No Distinction between ULFs and Filaments—The simple model considers four structural states, which are tetramers, octamers, hexadecamers, and ULFs. No distinction is made between ULFs and filaments. An ULF is regarded as the shortest filament possible that is generated. The formation of ULFs constitutes the first step in the three-phase model (16): two tetramers associate laterally into an octamer, two octamers then associate laterally to yield a hexadecamer; and eventually, two hexadecamers associate laterally into an ULF. Intermediates containing other numbers of tetramers, e.g. three or six, were not considered. Four state variables are defined to represent the four structural states: T, number concentration of tetramers; O, number concentration of octamers; H, number concentration of hexadecamers; and F, number concentration of filaments.

The simple model uses three kinetic rate constants. \( k_1 \) is the rate of lateral aggregation between intermediates (\( A_{11} \) interaction type, as previously reported in (14)), \( k_2 \) is the rate of longitudinal aggregation of a tetramer to the end of a filament, and \( k_3 \) is the rate of longitudinal aggregation of two filaments (end-to-end annealing of filaments).

From this the following rate equations are derived:

\[
\begin{align*}
\frac{dT}{dt} &= -2k_1T^2 - k_1TF \\
\frac{dO}{dt} &= k_1T^2 - 2k_2O^2 \\
\frac{dH}{dt} &= k_2O^2 - 2k_1H^2 \\
\frac{dF}{dt} &= k_1H^2 - k_3F^2
\end{align*}
\]

(Eq. 1) (Eq. 2) (Eq. 3) (Eq. 4)

The formalism used in the simple model allows expressing the MFL as,

\[
L_m(t) = \frac{c_0 - T(t) - 2O(t) - 4H(t) - 8U(t) + d_1 l_{ULF} U(t)}{d_2(F(t) + U(t))}
\]

(Eq. 5)

where \( corr \) is a correction factor taking into account the length reduction due to the aggregation of ULFs. As reported previously (23), a length reduction during assembly of ~20 nm can be detected using SFM. A value of 20 nm was used for \( corr \) to account for that effect. \( c_0 \) is the initial concentration of tetramers, and \( d_1 \) is the linear density (184 tetramers per micron of filament) of the vimentin filament (16). Hence, the dynamic variable \( L_m(t) \) represents the MFL at time \( t \).

Extended Model: ULFs and Filaments Are Distinct Species—The extended model assumes that the formation of a first elongated product results from the longitudinal annealing of two ULFs, and it distinguishes between the elongation of filaments by the addition of single tetramers, single ULFs, or end-to-end annealing of longer filaments. As ULFs and filaments are now distinct from each other, the definition of a fifth state variable is required: \( U \) is the number concentration of ULFs. Similarly, two extra kinetic parameters have to be defined: \( k_2 \) is the rate of longitudinal annealing of two ULFs (\( A_{22} \) interaction type (14)), and \( k_3 \) is the rate of longitudinal aggregation of an ULF to the end of a filament. The dynamics of tetramers, octamers, and hexadecamers are unchanged and are still governed by Equations 1–3, but the dynamics of the ULFs and filaments are now described as follows.

\[
\frac{dU}{dt} = k_1H^2 - 2k_2U^2 - k_3UF
\]

(Eq. 6)

\[
\frac{dF}{dt} = k_2U^2 - k_3F^2
\]

(Eq. 7)

By setting a kinetic parameter to zero in the system, the corresponding reaction is not considered. Hence to study all possible mechanisms for filament elongation, the parameters \( k_1, k_2, k_3 \) and \( k_3 \) related to tetramer, ULF and filament addition, respectively, are alternatively set to zero. Consequently, the extended model (Equations 1–3, 6, and 7) considers 2\(^5\) = 8 different scenarios for filament elongation (see Fig. 1). Although this model does not include a disassembly reaction at this stage, this can easily be added.

The MFL over time is now described by,

\[
L_m(t) = \frac{c_0 - T(t) - 2O(t) - 4H(t) - 8U(t) + d_1 l_{ULF} U(t)}{d_2(F(t) + U(t))}
\]

(Eq. 8)

where \( l_{ULF} \) is the length of an ULF. The extended model distinguishes between ULFs and filaments during the elongation; therefore the introduction of \( l_{ULF} \) was necessary to calculate the correct MFL.

Sensitivity Analysis

A sensitivity analysis was performed with the eight scenarios resulting from the extended model. The latter contains \( p = 5 \) parameters representing the reaction rates. For each scenario a system of \( n(p + 1) \) equations was constructed. Here \( n \) represents the number of equations in the model, i.e. the number of structural states considered, \( n = 5 \). The model equations (Equations 1–3, 6, and 7) can be rewritten as,

\[
\frac{dy}{dt} = f(t, y, p)
\]

(Eq. 9)

where \( f \) is the right-hand side vector of the equations, \( y \) is the five-vector of state variables (T, O, H, U, and F), and \( p_i \) is the five-vector of parameters \( (k_1, k_2, k_3, k_4, k_5) \). The sensitivities of structural states with respect to the parameter \( p_i \) are then defined as the five-vector satisfying the forward sensitivity equation,

\[
\frac{d}{dt} \frac{\partial y}{\partial p_i} = \frac{\partial f}{\partial y} \frac{\partial y}{\partial p_i} + \frac{df}{\partial p_i}
\]

(Eq. 10)

obtained by differentiating the original system (Equation 9) with respect to \( p_i \). The sensitivities, or the so-called sensitivity coefficients, are the derivatives of the model response with respect to the different parameters; they represent the rates of change of the structural state concentrations with respect to an increase in a given parameter \( p_i \). Sensitivity of the MFL to the parameter \( p_i \) is obtained by differentiating Equation 8 with respect to \( p_i \) as follows.

\[
\frac{\partial L_m(t)}{\partial p_i}
\]

(Eq. 11)

To compare sensitivities, the normalized coefficients were used,
Vimentin Assembly Kinetics

![Schematic representation depicting the hypotheses we considered for filament elongation, namely addition of tetramers, ULFs, and annealing of filament ends, in the extended model. The eight scenarios, denoted I–VIII, represent all combinations of the three modes of filament elongation mentioned. The kinetic rate constant for filament elongation by addition of tetramers is \( k_1 \), that for filament elongation by longitudinal annealing of ULFs to the end of a filament is \( k_2 \), and that for filament elongation by end-to-end annealing is \( k_3 \). A particular reaction will not occur if the respective rate constant is equal to zero (for example, if \( k_1 = 0 \), then no addition of tetramers will occur). The eight scenarios are investigated by mathematical modeling (see Equations 1–3 and 6–8 under “Experimental Procedures”). For all eight scenarios \( k_i, k_j \), and \( k_1 \), rate of lateral aggregation of tetramers; \( k_2 \), annealing of two ULFs to form a short filament) are assumed to be positive.

\[
p = \frac{p_1}{L_m(t)} \frac{\partial L_m(t)}{\partial t} \quad \text{(Eq. 12)}
\]

and the sensitivity coefficients were determined numerically.

**Data Fitting**

The temporal evolution of the MFL for each scenario (Fig. 1) of the extended model was used to fit the MFL observed in the SFM and EM experiments as a function of time (between 10 s and 20 min). Similarly, the dynamics of the five structural states obtained from the numerical solutions were used to fit the SFM and EM observations. This method allowed us to discriminate between the different scenarios to find the best one to describe the in vitro filament assembly kinetics. Moreover, this method provided estimates for the rate constants. The curve fitting work was done with Berkeley Madonna 8.0.6

**Statistical Methods**

For testing the similarity of the results of the MFL on the different specimen supports the Wilcoxon signed rank test was used. This test is a non-parametric test, which does not make any assumptions about the underlying distribution of the measurements. It was demonstrated to yield viable results also for smaller sample sizes. The test is implemented in the MatLab package and tests the equality of the medians of the samples. It calculates two values, \( p \) and \( h \); \( h \) is zero if the difference in the medians of the tested samples is not significantly different from zero. \( h \) is one if the two medians are significantly different. \( p \) is the probability of observing a result equally or more extreme than the one using the data, if the null hypothesis is true. \( p \) is calculated using the rank values for the differences between corresponding elements in the tested samples. If \( p \) is near zero, this casts doubts on the hypothesis of similarity of the tested samples.

In addition, we took the mean value of all measurements on one specimen support (mica, \( n = 1018 \); carbon grids, \( n = 3587 \); and glass, \( n = 1323 \)). These results, and the confidence intervals at 95%, were integrated into our calculations. In combination with the mean values and the confidence intervals for the individual measurements, this gave an additional measure for the reliability of the measurements on the respective supports (see Fig. 2).

**RESULTS AND DISCUSSION**

The Measured MFL Is Not Influenced by the Type of Specimen Support Medium and the Adsorption Time

Vimentin IF assembly was followed for up to 15 min by recording the MFL at different time points by EM and SFM. For both EM and SFM the filaments need to be immobilized on a solid support, a process that depends on the adhesive properties of the support (19). For EM, filament growth was stopped by glutaraldehyde fixation before an aliquot was adsorbed onto a carbon-coated EM copper grid and negatively stained with uranyl acetate (see “Experimental Procedures”). Similarly, for SFM filament growth was quenched by rapid dilution (1:80) of the assembly mixture followed by adsorption of an aliquot to either mica or glass (see “Experimental Procedures”).

For vimentin IFs, after 15 min of assembly at 37 °C, the measured MFL values obtained when adsorbed for different times to either carbon-coated copper grids (see Fig. 2A) or mica (see Fig. 2B) appeared rather similar and were closely distributed around the global mean length (see straight gray lines). In contrast, when adsorbed to glass the measured MFL values were more widely spread (see Fig. 2C). The Wilcoxon signed rank test confirmed that there is no significant difference between the results generated on carbon-coated grids and mica, respectively, at the 95% confidence interval (\( p = 1 \) and \( h = 0 \)). In contrast, the results obtained on glass were significantly different from those on mica or carbon films at the same confidence interval (\( p = 0.03 \) and \( h = 1 \)).

Taken together, SFM images of IFs adsorbed to glass supports should not be used for quantitative MFL measurements, particularly when the results are compared with those of IFs adsorbed to carbon films imaged by EM. Furthermore, for all subsequent experiments we chose a 2-min adsorption time both to carbon-coated copper grids and mica, because this yielded an optimal filament density for MFL measurements.

**Time Course of Vimentin IF Assembly Assessed by EM and SFM**

Vimentin IFs were assembled in vitro at 37 °C for 10 s to 20 min, and assembly reaction was quenched by preparing aliquots for EM or SFM (see above and “Experimental Procedures”). Hence, the resulting EM and SFM images depict the time-dependent elongation of vimentin IFs (see Fig. 3). The contour length of the adsorbed filaments was measured by

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the public domain software ImageJ (National Institutes of Health) (21), and from it the MFL was calculated for each time point (Table 1). Instead of the standard deviations, the confidence intervals at 95% are given. This is because the standard deviations became rather large for longer assembly times due to the high fluctuations in the length of the individual filaments (see Fig. 2).

Kinetic Modeling of in Vitro IF Assembly

Based on a qualitative description of the IF assembly process (16), two models were developed: a simple model and an extended model. Both models describe IF assembly as a result of ULF formation followed by filament elongation via longitudinal annealing of ULFs or longer filament. The extended model allows testing different pathways for the in vitro assembly of vimentin IFs. The different pathways are described by eight different scenarios (see Fig. 1). This then allowed determination of the most probable scenario as well as the influences of each reaction involved in the filament polymerization process. Last but not least, we estimated some of the reaction rates by fitting the model to the experimental data.

The Simple Model Is in Poor Agreement with the Experimental Data—As documented in Fig. 4, the early steep increase in the measured MFL cannot be explained by the simple model (see Equations 1–5 under “Experimental Procedures”). Nevertheless, the simple model was helpful in estimating a numerical value for the rate constant of ULF formation, \( k_1 \). Accordingly it is obvious from Fig. 4 that \( k_1 \) has to be in the range of \( 10^{-7} \text{ M}^{-1} \text{s}^{-1} \). If \( k_1 \) were much smaller, the number of ULFs formed would be too low, and hence the filament elongation would slow down. With a \( k_1 \) of \( 2 \times 10^{-7} \text{ M}^{-1} \text{s}^{-1} \), 50% of the ULFs were formed after 2 s. Based on this evaluation, we chose a \( k_1 \) of \( 2 \times 10^{-7} \text{ M}^{-1} \text{s}^{-1} \) for the fitting of the data in the latter model. This value is in agreement with an earlier observation documenting that 10 s after initiation of vimentin IF assembly ULFs are the predominant species. In addition, the simple model predicts

According to the distribution around the overall MFL, mica appears to be the most consistent surface over time. C, adsorption of the filaments onto glass and measurement of the filament length by SFM. The overall MFL on glass is 668 nm, and the MFL at different adsorption times vary considerably.
that the annealing of tetramers \(k_t\) to the ends of growing IFs represents an insignificant scenario for filament elongation.

**The Extended Model Agrees Well with the Experimental Data**—In contrast to the simple model, the extended model allows us to quantitatively assess the MFL dynamics (see Equation 8 under “Experimental Procedures”) on the basis of the eight different scenarios (see Fig. 1) for elongation of the filaments. As documented in Fig. 5, a numerical solution of the MFL for all eight scenarios (see Fig. 1) was calculated for a given set of parameters. Accordingly, the MFL dynamics for a given scenario can be classified into one of four different modes (Table 2): 1) Scenarios IV and VIII yielded very short filaments that did not seem to elongate further (see Fig. 5, dotted curve). 2) Scenarios II and VI produced filaments that were elongated. However, within the first 10 s of the assembly process a plateau was reached and no further elongation occurred (see Fig. 5, dashed curve). 3) Scenarios III and VII revealed filaments that exhibit linear growth from the first few seconds on (see Fig. 5, dashed-dotted curve). 4) Compared with the other ones, scenarios I and V formed fast-growing filaments during the first 10 s of the assembly process. This phase was followed by a decrease of the filament elongation rate. However, filament assembly reached a constant rate exhibiting a linear dependence between MFL and assembly time (see Fig. 5, solid curve).

By comparison with the experimental data (see Table 1), scenarios II, IV, VI, and VIII can be excluded. The filaments modeled by these scenarios are too short with a plateau being reached after a certain assembly time beyond which no further filament elongation occurred (see Table 2). Hence, the filament modeling by these scenarios does not match the observed in vitro filament elongation. The common requirement shared by these four scenarios is the absence of any end-to-end annealing of filaments \(k_t = 0\); see Fig. 1). Taken together, we conclude that end-to-end annealing of filaments is an essential process for filaments to grow to the observed MFL values. In contrast, scenarios III and VII assume end-to-end annealing of filaments without, however, any ULF addition and with tetramer addition being on or off, respectively. Finally, in scenarios I and V, filament elongation is driven by both ULF addition and end-to-end annealing. Like scenarios III and VII,
TABLE 2

Characterization and comparison of the eight different scenarios resulting from the extended model

The data depicted in Fig. 5 have been used to compare the eight scenarios defined in Fig. 1.

| Similar dynamics | Common hypotheses | Behavior | Validation |
|------------------|-------------------|----------|------------|
| Scenarios IV and VIII | Only tetramer addition | No elongation | No |
| Scenarios II and VI | ULF addition, and no end-to-end annealing | Fast stabilization of the mean length, short filaments | No |
| Scenarios III and VII | End-to-end annealing, and no ULF addition | Linear-like elongation | Problems at early stages of the process |
| Scenarios I and V | ULF addition, and end-to-end annealing | Fast elongation with decreasing rate of elongation | Yes |

TABLE 3

Normalized sensitivities of the structural state concentrations and the mean filament length, to the five parameters in scenarios I and III resulting from the extended model

| $k_1^{\prime}$ | I | III | $k_2$ | I | III | $k_3$ | I | III | $k_4$ | I | III | $k_5$ | I | III |
|--------------|---|-----|-------|---|-----|-------|---|-----|-------|---|-----|-------|---|-----|
| **T** | $-1$ | $-1$ | $0$ | $0$ | $0$ | $-10^{-2}$ | $0$ | $0$ | $10^{-4}$ | $10^{-3}$ |
| **O** | $-1$ to $-0.5$ | $-1$ | $0$ | $0$ | $-10^{-6}$ | $-10^{-6}$ | $-10^{-6}$ | $10^{-6}$ | $0$ | $10^{-5}$ | $10^{-5}$ |
| **H** | $-1$ to $0$ | $-1$ | $0$ | $0$ | $-10^{-4}$ | $-10^{-4}$ | $-10^{-4}$ | $10^{-4}$ | $0$ | $10^{-4}$ | $10^{-4}$ |
| **U** | $-0.3$ to $0.2$ | $-0.5$ to $0.2$ | $-10^{-2}$ to $0.15$ | $-10^{-4}$ to $0.7$ | $0$ | $0$ | $-10^{-4}$ | $-10^{-4}$ | $0$ | $10^{-4}$ | $10^{-4}$ |
| **F** | $-10^{-1}$ | $-10^{-1}$ | $-10^{-1}$ to $0.3$ | $-10^{-1}$ to $0.3$ | $0$ to $0.5$ | $0.5$ to $1$ | $0$ to $0.5$ | $0.5$ to $1$ | $0$ to $1$ |

**Notes:**
- $a$: Kinetic rate constants; $k_1$, rate of lateral aggregation of T, O, and H into ULFs; $k_2$, rate of longitudinal annealing of two ULFs; $k_3$, rate of tetramers annealing to filaments ends; $k_4$, rate of longitudinal growth via ULFs annealing to filament ends; $k_5$, rate of longitudinal growth via filament end-to-end annealing.
- I and III are abbreviations for scenarios I and III of the extended model.
- The rows list the influence of a change in a certain rate constant on the concentration of: T, tetramers; O, octamers; H, hexadecamers; U, ULFs; F, filaments; Lm, MFLs.
- Differences on the order of magnitudes between the scenarios.
- Differences on the order of magnitudes between the scenarios.
- Differences in the behavior between scenarios I and III.

scenarios I and V only differ from each other by the presence or absence of tetramer addition. Most likely, in these four scenarios tetramer addition has no significant effect on the MFL. Notice also that scenario VIII, which exhibited no elongation, yielded results that are similar to scenario IV, which assumes tetramer addition only.

Discrimination between the different potential modes of assembly exhibited by scenarios I, III, V, and VII was accomplished by focusing on the early stages of the respective elongation process. At the early stage, scenarios I and V revealed a sharp increase of their MFL, whereas the increase of the MFL in scenarios III and VII was significantly slower (see Fig. 5). However, scenarios III and VII were reaching a stable growth rate within their first 10 s of assembly. For comparison, the stabilization of the growth rates in scenarios I and V was reached later due to the presence of ULFs at the early stages of the experiment, which contributed to the filament elongation. Similar to scenarios III and VII, the time-dependent increase of the MFL became linear, too, in scenarios I and V after the annealing of ULFs was complete.

Similar to the experimental data (see Table 1), the dynamics exhibited by scenarios I and V yielded a steep increase of the MFL during the first few seconds of assembly followed by a slower increase. As documented in Table 2 and Fig. 5, fitting the numerical solutions of the eight different scenarios (see Fig. 1) to the experimental data confirms that scenarios I and V fit the measured filament assembly data best. Taken together, these findings clearly demonstrate that the process of filament elongation requires both ULF addition and end-to-end annealing of existing filaments to reproduce the measured data. Last but not least, tetramer addition does not appear to play any significant role during filament elongation.

### Testing the Effect of Parameter Variation by Sensitivity Analysis

The sensitivity coefficient is a measure for the influence of a given modeling parameter on the concentration of a particular reaction intermediate (see “Experimental Procedures”). Here, only the results for the sensitivity analysis of scenarios I and III are presented. Accordingly, the sensitivity coefficients were determined numerically and then normalized (Equation 12 and Table 3). Similar results were obtained for scenarios V and VII, but in these the sensitivity coefficients for $k_1$ were equal to zero (see Fig. 6). In both scenarios I and III, an increase in $k_1$ yielded either no effect or only an insignificant decrease of the concentrations of the four structural states, and it resulted in an insignificant increase of the MFL ($\sim 10^{-6}$-fold). Moreover, the sensitivity analysis confirms our earlier conclusions (see Kinetic modeling if in vitro IF assembly) that tetramer addition to the growing filaments represents an insignificant event in the course of filament elongation.

**The Soluble Pool of Tetramers**—This pool is affected similarly in scenarios I and III by each of the reactions (row “T”) in Table 3. The reaction that predominantly influences the soluble pool is the lateral aggregation reaction driven by the rate constant $k_1$. Similar conclusions hold for the octamer (row “O” in Table 3) and hexadecamer (row “H” in Table 3) populations.

**ULFs**—In scenarios involving ULF addition (i.e. I and V), lateral aggregation ($k_1$) of T, O, and H into ULF, filament nucleation ($k_4$) through longitudinal annealing of two ULFs, and ULF addition ($k_3$) to a growing filament had a negative effect on the ULF population (row “U” in Table 3), whereas end-to-end annealing had a positive influence. In scenarios with no ULF addition (i.e. III and VII), lateral aggregation of T, O, and H ($k_1$) may have either positive or negative effects depending on the
FIGURE 6. Normalized sensitivity coefficients (normalized according to Equation 12, sensitivity range from −1 to 1) of the filament number concentration F (in scenario I) to the rate $k_1$ computed for different values of $k_2$ and $k_u$ (varying from $0.001 \times 10^{-6} M^{-1} s^{-1}$ to $10 \times 10^{-6} M^{-1} s^{-1}$, and $k_u$ varying from $0.001 \times 10^{-6} M^{-1} s^{-1}$ to $10 \times 10^{-6} M^{-1} s^{-1}$). A sensitivity of 0.2 of a variable is interpreted as follows: an increase of 1 unit of a given parameter will yield a 20% increase of the corresponding variable. If the sensitivity is negative, then an increase of 1 unit of a given parameter will result in a 20% decrease of the variable value. The asterisk indicates that for $k_1$ and $k_u$ equal to $1 \times 10^{-6} M^{-1} s^{-1}$, an increase in $k_1$ will induce a decrease of the filament number of $-1\%$. The filled square indicates that, for $k_2 = 8$ and $k_u = 0.5 \times 10^{-6} M^{-1} s^{-1}$, an increase in $k_1$ will induce an increase of the filament number of $-1\%$.

values of the other parameters. Filament nucleation (see above) affects the ULF population negatively with a higher magnitude than in the case where ULF addition to growing filaments is considered. Furthermore, end-to-end annealing ($k_f$) of growing filaments yielded no significant change in the proportion of ULFs.

Filaments—Whatever scenario we consider an increase in $k_1$ or $k_2$ may have either a positive or negative effect on the number of filaments, depending on the values of the other parameters (row “F” in Table 3). The magnitude of this effect was amplified in the case of no ULF addition. With ULF addition, $k_2$ had a slight negative effect on the number of filaments. As expected, end-to-end annealing of growing filaments had a negative influence on the number of filaments. Consequently in scenarios combining all types of filament elongation (i.e. I and V), the only reaction that significantly influenced the number of long filaments is the end-to-end annealing ($k_f$) of growing filaments.

MFL—If scenarios involving both ULF and filament addition are considered (i.e. I and V), an increase in $k_1$ or $k_2$ had either a positive or a negative effect on the MFL, depending on the values of the other parameters (row “Lm” in Table 3). On the other hand, if ULF addition is not allowed, an increase in $k_1$ or $k_u$ only yielded a positive effect on the MFL. As expected, ULF addition, if allowed, and end-to-end annealing both had a positive effect on the MFL. However, an increase in $k_1$ had a stronger influence on the MFL than an increase in $k_u$.

In scenarios with no ULF addition to the growing filaments (i.e. III and VII), the principal reaction for filament elongation was end-to-end annealing of filaments. A similar conclusion is plausible for scenarios with ULF addition (i.e. I and V). Besides end-to-end annealing, ULF addition to growing filaments was the second most important reaction for filament elongation occurring at a slightly lower order of magnitude.

Fitting the Extended Model to the Experimental Data Allows an Estimate of the Apparent Reaction Constants

The kinetic simulation and data analysis program Berkeley Madonna 8.0 was used to fit the extended model to the experimental data. With regard to the results of the sensitivity analysis (see Table 3 and Fig. 6) and the numerical simulations (see Fig. 5 and Table 2) we decided to fit scenario V only to the...
experimental data. Although both scenario I and V could describe the experimental data appropriately, scenario I was not further pursued to minimize the number of variable parameters. As shown by sensitivity analysis and numerical solution of the different scenarios (see Fig. 5 and Table 2), tetramer addition played no significant role in filament elongation.

Scenario V was fitted to all experimental data sets (see Fig. 7). In contrast to scenario VII, which only used end-to-end annealing of filaments, scenario V also employed ULF addition to growing filaments. Moreover, scenario V reproduced the experimental data best, including the measurements covering the first few seconds. Overall, scenario VII, too, reproduced the experimental data fairly well, but a good fit was only obtained for the longer assembly times (not shown). During the first seconds to minutes the scenario VII failed to fit the increase in the MFL. In fact, without ULF addition to the filament ends, scenario VII yielded a slower than measured increase of the MFL during the early phases of the assembly process.

Taken together, we conclude that (i) the presence of ULFs and (ii) both ULF and end-to-end annealing of growing filaments are necessary to fit the experimental data with scenarios described by the extended model (see Figs. 1 and 5). In support of this, we have documented that annealing of only ULFs and/or addition of only tetramers to growing filaments cannot reproduce the MFLs measured during filament assembly (see Fig. 5, scenarios II, IV, and VI). The rate constants estimated for different vimentin concentrations, i.e. by fitting scenario V to the measured MFL data, are listed in Table 4. These data comprise

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the first quantitative description of the assembly reaction of human vimentin IFs in vitro.

**Implications for in Vivo Assembly**

For MTs and MFs, the investigation of the in vitro assembly reactions of their subunits, i.e. actin and tubulin, has provided extremely valuable insight into the dynamics of filaments as well as the principal molecular reactions leading to assembly or disassembly (24, 25). This work has also defined the limits of what can be learned from in vitro studies and that in living cells additional factors are important for assembly and stability of these filaments such as chaperones, associated proteins, organizing centers, and post-translational modifications. However, in contrast to actin and tubulin, cytoplasmic IF proteins refold properly after denaturation and assemble without the need for any cofactor. Moreover, although IF proteins are phosphorylated in vivo, the removal of these phosphate groups has little influence on their assembly properties.

The results of our study indicate that IFs can elongate by end-to-end annealing of filaments, and this may indeed also be of relevance for the in vivo situation. Employing green fluorescent protein-tagged proteins, it has been observed that vimentin filament network formation is proceeding by fusion reactions of “dots,” i.e. structures below the optical resolution (0.2 μm) in the light microscope, to short filaments, termed squiggles, which may grow further during transport by MT-associated motor complexes. At the cell periphery they are then integrated into the existing filament system by fusion to filament ends (28, 29). Similarly, the neuronal IF protein peripherin is first assembled into short filaments fragments that are transported along the axon and then incorporated into longer filaments (30, 31). Hence, these in vivo intermediates called dots or “short fragments” are probably ULFs or ULF-like structures. Moreover, the observation that newly formed filaments are able to integrate into existing filament networks indicates that the end-to-end annealing deduced from our models (Fig. 8) does indeed take place in vivo.

**Conclusions**

Our experimental data and the mathematical model derived from it are in agreement with the scenario that both ULF addition to and end-to-end annealing of growing filaments represent the prevailing mechanisms driving IF elongation. Furthermore, exactly as was previously observed experimentally (9, 15),
our model suggests that ULFs are the major, productive intermediates during the early stages of filament assembly. In our in vitro assembly regime we start from tetramers (14), which appears rather plausible in view of the fact that tetramer complexes of vimentin have indeed been demonstrated in extracts obtained from cultured cells by sucrose density gradient centrifugation (32). As yet, our experiments cannot resolve the intermediate reactions occurring in the sub-second range after initiation of assembly (15). To this end, recent studies of vimentin assembly by small-angle x-ray scattering show the existence of octameric complexes as intermediates during ULF formation when starting from tetramers (33). Based on these results, IF assembly may be described to proceed via formation of ULFs within the first few seconds of assembly (see Fig. 8A) followed by the filament elongation, which is predominantly driven by the end-to-end annealing of ULFs and subsequent end-to-end annealing of more extended filaments (see Fig. 8, B and C). The next challenge will be to further enhance the model so that not only the MFL is modeled but the length distribution of the filaments will be correctly predicted for a given assembly time.

Assessing the filament assembly in such a way will provide a more flexible and accurate criterion for the comparison between the model and the experimental results. Having a robust assembly model at hand, we will then be in a better position to more rationally understand the effect of the rapidly growing number of IF protein mutations on filament formation, including those found in keratins, desmins, glial acidic fibrillary protein, the neurofilament proteins, and nuclear lamin A. In humans most of these mutations slowly but definitely give rise to severe, mostly incurable disease phenotypes (12, 34, 35). As encountered in recent studies on desmin-related myopathies, a structural insight into the assembly of the mutant protein, in particular, when compared with the corresponding wild-type isofrom, is fundamental to derive a solid concept for the description of the respective pathomechanism (17, 18, 36, 37).

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