Site-directed Spin Labeling and Electron Paramagnetic Resonance Determination of Vimentin Head Domain Structure

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Atya Aziz, John F. Hess, Madhu S. Budamagunta, John C. Voss, and Paul G. Fitzgerald*†

From the Departments of Cell Biology and Human Anatomy and Biochemistry and Molecular Medicine, School of Medicine, University of California, Davis, California 95616

Intermediate filament (IF) proteins have been predicted to have a conserved tripartite domain structure consisting of a largely α-helical central rod domain, flanked by head and tail domains. However, crystal structures have not been reported for any IF or IF protein. Although progress has been made in determining central rod domain structure, no structural data have been reported for either the head or tail domains. We used site-directed spin labeling and electron paramagnetic resonance to analyze 45 different spin labeled mutants spanning the head domain of vimentin. The data, combined with results from a directed spin labeling and electron paramagnetic resonance to analyze 45 different spin labeled mutants spanning the head domain of vimentin. The data, combined with results from a previous study, provide strong evidence that the polypeptide backbones of the head domains form a symmetric dimer of closely apposed backbones that fold back onto the rod domain, imparting an asymmetry to the dimer. By following the behavior of spin labels during the process of in vitro assembly, we show that head domain structure is dynamic, changing as a result of filament assembly. Finally, because the vimentin head domain is the major site of the phosphorylation that induces disassembly at mitosis, we studied the effects of phosphorylation on head domain structure and demonstrate that phosphorylation drives specific head domain regions apart. These data provide the first evidence-based model of IF head domain structure.

Intermediate filaments (IFs) are one of the three major classes of filamentous cytoskeleton. The IF gene family includes more than 65 different members, each expressed in a cell-specific or differentiation stage-specific manner. Most IFs, however, are assembled from only one to three IF proteins (1–4).

Mutations in IF genes have been implicated in more than 85 different human diseases, with most diseases resulting from similar changes to highly conserved amino acids (6). However, the capacity to develop a mechanistic understanding of the pathogenesis of these diseases or even of normal IF function has been limited by the inability to produce crystals suitable for solution of IF or IF protein structure. IF proteins can be solubilized in chaotropes such as 8 M urea, but upon dialysis into physiologic conditions, they spontaneously assemble into IFs. These properties, which are likely critical to the strength and stability of IFs, have apparently also confounded the production of useful crystals. Thus, efforts to establish IF structure have taken a variety of alternative approaches.

IF proteins show a wide range of size and a large divergence in primary sequence. They are united into a family, however, on the basis of several features. Among these features are: (a) cytoplasmic IF proteins are typically found in 8–11 nm IFs, and (b) in silico analysis of primary sequences predicts that all IF proteins possess a tripartite domain structure consisting of a central rod domain flanked by head and tail domains (see Fig. 5a).

As first predicted more than 20 years ago, the central rod domain has an overall length of ~310 amino acids and largely conforms to a heptad repeat pattern, suggesting the ability to form an α-helical coiled-coil dimer (“coil” domains) (5, 7–12). In contrast to other structural proteins, such as myosin, the central α-helical domain of IFs is interrupted by short sequences that do not conform to the heptad repeat pattern. As such, the individual coil domains are connected by regions of unknown structure, termed “linkers.” The size of the rod domain, the number and size of both coil and linker domains, as well as several other predicted structural features are well conserved among essentially all cytoplasmic IF proteins.

Many elements of the rod domain model have been tested using several different approaches, including cross-linking, crystallization of vimentin fragments, and cryo-electron microscopy. Most of these data support and refine a model of an in-register, in-parallel, coiled-coil dimer as the fundamental building block of the IF (13–21). These dimers assemble through various stages into 8–11 nm filaments, thought to be 16 dimers in cross-section.

A lot of data exist that document the contribution of head and tail domains to IF assembly (22–34). Other studies have identified multiple phosphorylation sites within the head/tail domains, and the role they play regulating assembly/disassembly of IFs (35–40). However, despite nearly 30 years of investigation, no evidence-based model of head or tail structure has emerged.

We have explored the utility of site-directed spin labeling and electron paramagnetic resonance (SDSL EPR) in the elucidation of IF structure. In this approach, a cysteine residue is placed at a specific site within the recombinant human IF protein vimentin, followed by covalent attachment of a small, amino acid-sized spin label to the cysteine. The modified protein is solubilized in urea, assembled into IFs by dialysis into physiologic buffer, and then analyzed by EPR spectroscopy. The resulting spectra yield information about backbone flexibility but also about the distance between spin labels in the range of...
0.8–2.1 nm. Although a given spin mutant yields only very local structure within the complex, a series of mutants can be used to assemble a much more comprehensive view of a given protein or macromolecular complex. Using this approach, we have been able to define \( \alpha \)-helical coiled-coil domains, establish spatial relationships between monomers in a dimer, and establish relationships between dimers in tetramers (specifically points of overlap and relative parallel/anti-parallel orientation), as well as the impact of disease-causing mutations and phosphorylation on IF head domain. The data permit the development of the first model of head domain structure for any IF protein. We also show some of the effects of both assembly and phosphorylation on IF head domain structure.

**EXPERIMENTAL PROCEDURES**

*Spin Labeled Proteins—Characterization, cloning, overexpression, purification, and spin labeling were done as described in detail in our previous reports (42, 44). In brief, spin labeled mutants were produced by substituting a cysteine residue at a series of different sites in the human vimentin head region using the mutagenic oligonucleotides and i Proof polymerase (Bio-Rad). Sequence changes were verified by automated DNA sequencing. Vimentin mutants were produced by bacterial overexpression using arabinose-inducible BL21 (DE3) cells (Invitrogen). Inclusion bodies were purified using lysozyme/DNase, high/low salt washes, and chromatography using fast protein liquid chromatography system (Akta fast protein liquid chromatography; GE Healthcare). The fractions were analyzed by SDS-PAGE, and peak fractions were pooled. Unless otherwise indicated, attachment of the nitroxide spin label to the cysteine was achieved by treating the purified protein liquid chromatography over a Source column (GE Healthcare). Protein concentrations were measured by the Bradford method. All other material was dialyzed overnight against different conditions that required dithiothreitol, the protein was spin labeled with 500 \( \mu \text{M} \) 3-malemidoproxyl (253375; Sigma-Aldrich). This was necessary because the dithiothreitol used in the phosphokinase reaction conditions would have released the methanethiosulfonate spin label, which is coupled to the cysteine via a disulfide bond, whereas 3-malemidoproxyl spin label is attached by alkylation of a thiol that is not affected by dithiothreitol. Following electron microscopy to verify the presence of filaments, the samples were treated with protein kinase A (catalytic subunit from bovine heart; Sigma-Aldrich) as previously reported (45) Briefly, protein (~2 mg/ml) was dialyzed overnight against kinase reaction buffer/assembly buffer (20 mM Hepes, 60 mM NaCl, 2 mM MgCl\(_2\), 6 mM EGTA) at room temperature. A 20-\( \mu \text{l} \) volume of vimentin filaments was incubated for 3 h at 30°C with (a) 10 \( \mu \text{l} \) of assembly buffer (control), (b) 5 \( \mu \text{l} \) of 10 mM ATP and 5 \( \mu \text{l} \) of assembly buffer (ATP only), (c) 5 \( \mu \text{l} \) of protein kinase A and 5 \( \mu \text{l} \) of assembly buffer (kinase only), or (d) 5 \( \mu \text{l} \) of protein kinase A and 5 \( \mu \text{l} \) of 10 mM ATP (kinase + ATP). After the incubation, a 5-\( \mu \text{l} \) sample was re-evaluated for filament presence/absence by electron microscopy and then analyzed by EPR.

**RESULTS**

For the sake of reference, the tripartite domain structure of vimentin, as predicted by the coiled-coil analysis method of Lupas *et al.* (49), is shown in Fig. 5a. The figure identifies the predicted head, rod, coil, linker, and tail domains. The residues analyzed in this report (residues 1–108) span the full length of the predicted head domain.

To investigate the proximity of vimentin head positions at the early stage of assembly, low salt dialysis was used to constrain the assembly of vimentin to protofilaments, an assembly intermediate thought to be a tetramer or slightly larger species (20, 50, 51). Because this protofilament species is thought to consist of the anti-parallel association of two parallel dimers...
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(see Fig. 5b), it facilitates the recognition of in-register spin coupling between sites in the vimentin dimer. The room temperature EPR spectra for the series of site-directed spin labels in the head domain of vimentin are shown in Fig. 1. The amplitude of each spectrum is plotted according to its normalized number of spins by double integration of the SDS-solubilized sample. Inset: the line width ratio, and is shown on a model spectrum. This ratio, calculated from the spectra acquired at −100 °C, reflects the dipolar interaction strength and thus distance between spin labels.

Although it is obvious that dipolar coupling dominates the broadening of some spectra (e.g. position 9), a model-independent evaluation of the dipolar contribution to the broadening can be obtained by comparing all positions in the absence of motion (at −100 °C). In this state the influence of motional averaging of the hyperfine anisotropy can be eliminated. The degree of dipolar broadening in the frozen spectra can be obtained from the ratio in the low temperature spectra (Fig. 1, inset), which yields a semi-quantitative estimate of distance between spin labels when the probes reside within 2 nm of each other. Thus, to ask whether the head domains were aligned in parallel or whether they diverged from one another, we scored each position within the head domain series according to its value (Table 1). This was done for both full-length IFs (IF) and the protofilaments that result from dialysis into low salt buffer (protofilament d/d). In intact filaments, 42 of the 45 mutants yielded d/d ratios of >0.39 or greater, indicating that the spin labels were within 2.1 nm of one another. Although our investigation of this region contains four small gaps (residues 18–21, 35–39, 66–72, and 77–82), these results provide evidence of a generalized dipolar coupling of spin labels placed at nearly any position within the vimentin head domain. Of the measured sites, the longest stretch for which the d/d shows low interaction (increased distance) is found in the region encompassing positions 91–101. This constitutes very strong evidence for a sustained proximity of the two polypeptide backbones along the full length of the head domain and leaves minimal opportunity for major divergence of the backbones at any point.

To explore whether the observed interaction between a given pair of spin labels represented the closest point of interaction, we mixed proteins labeled at different sites. Specifically, we mixed protein labeled at a given site (site a) with an equimolar amount of protein labeled at a slightly “upstream” site (site b) or a slightly “downstream” site (site c). This should yield a mixture of species, 50% of which is in an a-b configuration, 25% a-a, and 25% b-b. If the spin labels at site a are directly opposite one another, then such a mixture should show a drop in the d/d value because the high a-a value will be diluted by the lower and more abundant a-b value. If the a-b spins are closer together than the a-a, then the d/d value would increase, because 50% of the d/d value would be derived from spin pairs that are closer together. In all cases where we mixed spin labeled proteins with protein labeled at either an upstream or a downstream site, the
When purified vimentin is dialyzed from 8M urea into low salt buffer, assembly ceases at the protofilament stage, an intermediate in the \textit{in vitro} assembly process. The addition of assembly buffer causes very rapid assembly of \textit{bona fide} IFs from these protofilaments. To identify changes that may occur in the head domain during IF elongation/assembly, we examined the EPR line shapes of vimentin protofilaments and compared them with the spectra generated in full-length IFs. For the majority of positions within the head domain, little to no difference in the line shape is observed (not shown) between protofilaments and full-length filaments, indicating that the fundamental structure of the head domain is acquired by the protofilament stage of assembly. However, the region encompassing residues 50–60 experiences substantially higher dynamics in the protofilament stage, as reflected in a narrower EPR spectrum under low salt assembly conditions. The positions showing the most pronounced spectral differences at low salt are shown in Fig. 2. The amplitude of each spectrum in Fig. 2 is significantly higher in the low salt state. For sites within positions 50–60, each of the three lines in the EPR spectrum are narrowed in shape, demonstrating a shift toward higher motional freedom of the spin label.

It is also evident that the higher spectral amplitude of the low salt spectra within the 50–60 sequence may not result exclusively from increased motional freedom but may also reflect decreased dipolar interaction in the protofilament state. In fact, although positions 104 and 108 display a substantially higher spectral amplitude at low salt, their line shape difference between the two states is less pronounced (Fig. 2). Therefore, it is likely that the broadening of positions 104 and 108 in the IF state is largely due to dipolar coupling established by the proximity of their spins. To evaluate the broadening independent of spin label dynamics, the average distance separating the labels in the different vimentin assembly states was also determined.

### TABLE 1

| RESIDUE | Native Residue | Protofilament ($d_1/d$) | IF ($d_1/d$) | Distance Shift | EPR Structure |
|---------|----------------|-------------------------|--------------|---------------|---------------|
| 4       | R              | 0.42                    | 0.43         | 0.01          | IFs           |
| 5       | S              | 0.42                    | 0.44         | 0.02          | IFs           |
| 7       | S              | 0.41                    | 0.42         | 0.01          | IFs           |
| 8       | S              | 0.4                     | 0.38         |               | IFs           |
| 9       | S              | 0.43                    | 0.43         | 0             | Short IFs     |
| 10      | S              | 0.42                    | 0.37         |               | IFs           |
| 12      | R              | 0.48                    | 0.49         | 0.01          | IFs           |
| 13      | R              | 0.45                    | 0.46         | 0.01          | IFs           |
| 16      | G              | 0.4                      | 0.45         | 0.05          | IFs           |
| 17      | G              | 0.37                    | 0.41         | 0.04          | IFs           |
| 22      | S              | 0.45                    | 0.38         |               | IFs           |
| 27      | S              | 0.44                    | 0.41         |               | IFs           |
| 28      | R              | 0.46                    | 0.48         | 0.02          | UFs           |
| 29      | S              | 0.46                    | 0.46         | 0.02          | Short IFs     |
| 32      | T              | 0.53                    | 0.56         | 0.03          | IFs           |
| 34      | S              | 0.45                    | 0.47         | 0.02          | IFs           |
| 40      | L              | 0.4                      | 0.45         | 0.05          | IFs           |
| 43      | A              | 0.39                    | 0.41         | 0.02          | IFs           |
| 45      | R              | 0.41                    | 0.45         | 0.04          | IFs           |
| 48      | T              | 0.42                    | 0.42         | 0            | IFs           |
| 49      | S              | 0.39                    | 0.39         | 0            | IFs           |
| 50      | R              | 0.39                    | 0.44         | 0.05          | IFs           |
| 52      | L              | 0.39                    | 0.47         | 0.08          | IFs           |
| 54      | A              | 0.4                      | 0.41         | 0.01          | IFs           |
| 57      | P              | 0.37                    | 0.49         | 0.12          | IFs           |
| 60      | V              | 0.4                      | 0.58         | 0.18          | IFs           |
| 65      | S              | 0.42                    | 0.42         | 0           | IFs           |
| 73      | S              | 0.41                    | 0.46         | 0.05          | IFs           |
| 76      | G              | 0.43                    | 0.46         | 0.03          | IFs           |
| 83      | S              | 0.41                    | 0.43         | 0.02          | IFs           |
| 86      | F              | 0.46                    | 0.45         | 0            | IFs           |
| 88      | L              | 0.42                    | 0.48         | 0.06          | IFs           |
| 90      | D              | 0.48                    | 0.46         | 0            | IFs           |
| 91      | A              | 0.38                    | 0.41         | 0.03          | IFs           |
| 93      | N              | 0.41                    | 0.41         | 0            | IFs           |
| 97      | K              | 0.39                    | 0.41         | 0.02          | Short IFs     |
| 100     | R              | 0.42                    | 0.41         | 0            | IFs           |
| 101     | T              | 0.38                    | 0.4         | 0.02          | IFs           |
| 102     | N              | 0.45                    | 0.48         | 0.03          | IFs           |
| 103     | E              | 0.52                    | 0.55         | 0.03          | IFs           |
| 104     | K              | 0.43                    | 0.5         | 0.07          | IFs           |
| 105     | V              | 0.37                    | 0.4         | 0.03          | IFs           |
| 106     | E              | 0.37                    | 0.43         | 0.06          | IFs           |
| 107     | L              | 0.52                    | 0.63         | 0.11          | UFs           |
| 108     | Q              | 0.38                    | 0.43         | 0.05          | Short IFs     |

FIGURE 2. Intermediate filaments versus protofilaments. Room temperature EPR spectra were gathered from intact intermediate filaments (IF, red spectra) and from protofilament subunits (black spectra) generated by dialysis against a low salt (LS) buffer. The number at the left of each pair of spectra identifies the amino acid residue where the spin labeled cysteine residue is located. Spin labels showed a strong mobility shift as the sample transitioned from protofilamentous stage to intact IFs. The $d_1/d$ values from spectra gathered at $-100^\circ$C are shown in parentheses.
by comparing the $d_i/d$ values from samples at $-100\,^{\circ}\mathrm{C}$ (Table 1). The difference between the protofilament and intact filament values are indicated as the distance shift in Table 1. At several sites the distance shift is large, suggesting that the distance between spin labels and by extrapolation that between the head domain backbones change as filament assembly proceeds.

Interestingly, positions showing the largest change in spectral shape when the sample assembles from the protofilament to the IF stage cluster into two regions, positions 50–60 and 104–108 (Table 1 and Fig. 2). These two regions also display the largest distance shift, indicating that these sites are on average, closer in the assembled filament than in the protofilament. For example, at sites 50 and 106 there is no detectable interaction in the protofilament stage, but these sites transform into strongly interacting sites when assembled into IFs. The spectra shown in Fig. 2 undergo a minimum difference of 0.05 in the distance shift upon IF assembly, with position 57 showing the greatest shift (0.12). The sole exception outside these two regions is position 28, which shows a significant decrease in side chain dynamics but displays a close proximity in both the protofilament and IF states (Table 1 and Fig. 2).

Formation of IFs by dialysis in vitro has been shown to proceed in a sequential manner; protein monomers assemble into dimers, followed by assembly of dimers into tetramers where rod domains 1 overlap (A11 configuration), followed by an unknown structure where rod domains 2 overlap in tetrameric interactions (A22 interactions), to higher order multimers, and finally to an intact filament that is estimated to be 16 dimers in cross-section. Previously, we have shown that at 6 M urea, initiation of the $\alpha$-helical backbone occurs, with progressive backbone stabilization and formation of a coiled-coil as the sample is dialyzed from 6 to 4 M urea, followed by tight packing of A11 and A22 tetramers by 2 M urea concentration. Fig. 3 shows similar experiments on proteins with spin labels at positions 12, 32, 83, and 86. All four positions display an increase in spectral broadening when the urea is removed from 8 to 2 M. The spectral changes observed here are similar to the changes we have previously reported in the spectral broadening of the 291 residue of linker L2 region 44. This early appearance of spin-spin interactions shows that the alignment of head domains in a dimer occurs very early in the assembly process.

Post-translational modification of the head domain by phosphorylation has been shown to disrupt the assembled IF in vitro and in vivo (35, 37–40). In previously published reports we have used SDSL EPR to show structural changes in vimentin that result from phosphorylation (45, 46). To explore the impact of phosphorylation further, we labeled positions 12, 32, and 83 using the maleimide spin label, because the disulfide bond tethering the (1-oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-methyl) methanethiosulfonate label is readily reduced by the dithiothreitol required to maintain active kinase enzyme (45). The 3-maleimidopropyl samples were then dialyzed overnight against kinase (protein kinase A) buffer. The assembly status of the protein was checked by electron microscopy before and after the kinase treatment. As a result of kinase treatment, the filaments were dissociated as confirmed by electron microscopy. The kinase-treated and untreated samples were then analyzed by EPR spectroscopy at both room temperature and $-100\,^{\circ}\mathrm{C}$. The room temperature spectra of positions 12, 32, and 83 are shown in Fig. 4. The post-phosphorylation spectra of all three positions show spectral line shapes that reflect substantially higher motional freedom following phosphorylation. The gain in amplitude (reduced broadening) of these spectral lines is consistent with a reduced contact between the two head domains in the dimer. To test whether this loss in broadening may also arise from an increased distance between head domains, we analyzed the samples in the absence of molecular motion by freezing the samples at $-100\,^{\circ}\mathrm{C}$. As shown in Fig. 4 (values in parentheses), the difference in the magnitude of the dipolar broadening ($d_i/d$) for samples treated with kinase indi-
The data provided here suggest that the head domains of a vimentin dimer form a symmetric structure, where the head domains are aligned in parallel and in register and where the protein backbones travel in close proximity for essentially their entire length. There are three key pieces of evidence provided in support of this model: (a) Spin labels at 42 different sites along the full length of the head domain backbones showed at least some degree of spin-spin interaction (a $d_1/d$ ratio of 0.39 or greater), indicating that the spin labels were located within 2.1 nanometers of one another at 42 different sites that span the full length of the predicted head domain. (b) Although there is fluctuation in the $d_1/d$ values along the length of the backbone, consistent with minor divergence and convergence of the backbones, the gap between interacting spin labels never exceeds 8 amino acids, an arrangement that likely precludes the existence of any significant structure that is not paired with structure from the adjacent head. Even when spin labels are more than 2 nm apart, the backbone positions of the adjacent chains may in fact be closer, because less structured folds may direct the side chains in opposite orientations and/or favor anomers of the spin labeled side chain that orient away from the dimer interface. Such variance in the position of the nitroxyl moiety of the probe relative to the backbone is expected to lead to a greater apparent distance between backbone atoms than is the case. (c) The mixing of proteins labeled at one site, with proteins labeled slightly upstream or downstream from that site, all suggest that the two head domains are aligned in register.

In a previous report we showed that residue 17 of the head domain is located in close proximity (<2.1 nm) to residue 137 of rod domain 1A. This establishes that the dimeric head domain folds back onto the rod, diminishing the possible contribution of head domain to dimer length by a minimum of 50%. This also suggests that the vimentin dimer has a polarity or “sidedness” with the head domain positioned along one side of the rod, but not the other. Collectively, these data provide strong support for a model of the vimentin head domains as a symmetric dimer, where the polypeptide backbones travel largely in parallel and in close proximity and fold back along one surface of the rod domain (Fig. 5a). Such a configuration is likely to impose significant constraints on the number of possible interactions that a given dimer head domain may have with adjacent dimers in the assembled filament.

In Fig. 2, we compare the EPR spectra for selected sites as the protofilaments assemble into filaments. These spectra indicate that the polypeptide backbones at these sites undergo...
large changes in mobility and proximity upon assembly, suggesting that that the head domain structure is dynamic and changes with assembly, becoming more compacted and motionally constrained.

Because phosphorylation has been demonstrated to regulate the assembly/disassembly of vimentin during physiologic processes such as mitosis, we have also used SDSL EPR to study structural changes in the head domain that occur with phosphorylation. We noted that phosphorylation results in an increase in the distance between head domains in at least three sites (residues 12, 32, and 83). In a previous report, we showed that phosphorylation induced an increase in distance between residues 17 of the head but not between residues 137 of the rod or between the sites where head and rod interact (17–137). Our data collectively support a model where head domains are positioned asymmetrically along one surface of the rod. This may result in two classes of dimer-dimer interactions, depicted by brackets A and B in Fig. 5b. In configuration A, the head domains (and thus the sites of phosphorylation) are concentrated between two dimers, whereas in configuration B, the head domains are oriented away from the dimer-dimer interface. Thus, the phosphorylation-induced separation of head domains is likely to selectively influence only a subset of dimer-dimer interactions (e.g. configuration A) rather than affecting all dimer-dimer interactions equally. This asymmetry may well explain why phosphorylation results in tetrameric subunits and not complete disassembly of IFs into monomers or dimers.

It will prove interesting to compare the head domain structure that we have established here in the Type III IF protein vimentin with that of the keratins. The Type III IF proteins (e.g. desmin, vimentin, glial fibrillary acidic protein, and peripherin) are homopolymeric, so the head domains are identical in both size and sequence. In contrast, the keratins form an initial heterodimer, consisting of Type I and Type II cytokeratins, where both the size and sequence of the head domains are different. Also, Steinert et al. (52) have noted the conserved presence of large numbers of glycines in the keratin head domains, which are often clustered. On the basis of this they have hypothesized the presence of glycine loops, glycine-rich regions that form flexible loops, interrupted by regions possessing aromatic residues that may tie the head domains together on the basis of hydrophobic interactions. Our SDS EPR data are consistent with hydrophobic amino acids in the head being in close proximity, likely because of hydrophobic interactions. In contrast to the keratins, however, vimentin (and the Type III IF proteins in general) have far fewer head domain glycines, with no more than two clustered adjacent to one another, and these glycines are not highly conserved. These differences may well portend some fundamental class differences in head domain structures among IF proteins.

This report presents the first evidence-based structural model for the head domain of any IF protein. We provide evidence that shows a pairing of adjacent head domains early in assembly. The head region displays mostly ordered structure, becoming more compacted with assembly, becoming more compacted and motionally constrained. Such sites may prove useful in assessing the process of IF assembly. In addition, we have identified three sites that reflect an increase in distance that occurs between head domains upon with phosphorylation, a physiologic regulator of vimentin assembly/disassembly. These same molecular markers also report a major increase in dynamics, indicating that phosphorylation results in a disruption beyond the level of quaternary structure. These data suggest a mechanistic model where phosphorylation-induced repulsion between two head domains in a dimer provides or at least contributes to the motive force that drives the head domains apart.

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