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The two coiled coils in the isolated rod domain of the intermediate filament protein desmin are staggered

A hydrodynamic analysis of tetramers and dimers

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Desmin protofilaments and the proteolytically derived α-helical rod domain have been characterized by high-resolution gel permeation chromatography (GPC) using columns calibrated for the determination of viscosity radii. Additional characterization by chemical cross-linking and the determination of sedimentation values allowed the calculation of the molecular dimensions of the molecular species isolated. In dilute buffers GPC separated desmin rod preparations into two complexes: a dimer species (single coiled coil) with a length of 50 ± 5 nm and a tetramer species (two coiled coils) with a length of 65 ± 5 nm. Thus the two coiled coils in the tetramer are staggered by approximately 15 nm. The hydrodynamically derived lengths of the rod dimer and tetramer are supported by electron microscopy after metal shadowing. The hydrodynamic properties of desmin protofilaments follow that of the rod tetramer. The data on the hydrodynamic analysis of the rod tetramer of desmin in solution are in full agreement with the structural information recently deduced from paracrystals of the rod of glial fibrillary acidic protein [Stewart, M., Quinlan, R. A. & Moir, R. D. (1989) *J. Cell Biol. 109*, 225 – 234]. Our results explain the inhomogeneity of molecules encountered in previous electron microscopical analyses.

The cytoplasmic intermediate filaments of vertebrates are built by common structural principles (reviewed in [1 – 3]). In the central α-helical rod domain, consisting of approximately 310 amino acids, about 280 residues are in α-helical conformation. The rod is flanked at both ends by shorter non-helical regions: the N-terminal head and the C-terminal tail domains. The rod is organized from double-stranded coiled coils [4] in which the participating α-helical chains are aligned in parallel and in register [5, 6]. Usually two coiled coils are found tightly associated in the tetrameric protofilament unit, which is the building block of the intermediate filaments [4, 7 – 10].

Two main questions arise about the structure of the tetramer: are the two coiled coils of the tetramer arranged in parallel or antiparallel manner and are the coiled coils aligned in register or with a stagger? Protofilaments and the isolated rod, lacking the head and tail domains due to limited proteolysis, have been extensively studied by electron microscopy [10 – 15]. Molecules displaying the unit length of the coiled coil (about 40 – 50 nm) as well as particles with the one and a half fold length (about 70 nm) have been recognized in these studies. Because a firm correlation of the observed length variants with the number of chains per particle was not reached, the mode of alignment remained unresolved.

We have now re-investigated the shape of the tetramer by hydrodynamic identification of distinct oligomer species. We show that the chymotryptically derived rod domain of chicken desmin (residues 70 – 415) is present in dilute buffers as a mixture of dimers (single coiled coils) and tetramers (double coiled coils), which can be separated by gel permeation chromatography (GPC). The chromatographically derived viscosity radii combined with sedimentation coefficients and molecular masses allowed the calculation of the molecular shapes for the isolated complexes. The hydrodynamically derived lengths of 40 ± 5 nm for dimers and 71 ± 5 nm for tetramers are supported by electron microscopy of the separated molecule species (50 ± 5 and 65 ± 5 nm). Application of the same hydrodynamic methods to the desmin protofilaments leads to the conclusion that they, unlike the rod, are present mainly as tetramers. Their hydrodynamically derived length is 70 ± 5 nm and thus similar to the length of the rod tetramer. If this means that they display the same spatial organization as the rod tetramer, then the two coiled coils of the protofilament should also be staggered by approximately 15 nm.

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Abbreviation. GPC, gel permeation chromatography.

MATERIALS AND METHODS

Protein isolation and rod preparation

Desmin from chicken gizzard was prepared as described [16]. The rod domain comprising residues 70 – 415 was obtained by digestion of protofilaments with chymotrypsin. It was purified by chromatography in 8 M urea buffer as described in detail before [14]. Renaturation of desmin and its rod was achieved by dialyzing the proteins from 8 M urea buffer, usually at concentrations around 0.5 mg/ml, against the appropriate gel permeation buffers (see below). Dialysis was at 4 °C for 12 h.

Gel permeation chromatography

GPC was performed on TSK 5000 PW columns (7.5 × 600 mm, LKB, Bromma, Sweden) at a constant flow rate of 5 ml/h at room temperature. Elution profiles were

monitored at 230 nm with a variable wavelength monitor (Knauer, Berlin). Most runs were performed in Tris/HCl pH 8.0 with different concentrations ranging between 3–10 mM Tris. Additional runs were in 10 mM Tris buffer with NaCl up to 0.17 M. Other buffers used were triethanolamine/HCl pH 8.0 and ethanolamine/HCl pH 8.0 (concentrations of both between 5–10 mM). All chromatography buffers contained 0.35 mM 2-mercaptoethanol and were continuously purged with helium. Marker proteins used for the calibration of the column were those contained in the gel filtration standard kit from BioRad (Richmond, CA, USA). Additionally tobacco mosaic virus (TMV) and bacteriophage MS2 were used. Sample volumes applied to the column were kept constant at 100 µl for the analytical runs. In some preparative runs, 200-µl samples were applied. The concentrations of the proteins or fragments applied to the column were kept in the range of 0.5 mg/ml. Samples used for electron microscopy or sedimentation analysis were rechromatographed after the measurements were made to ensure the identity with the original probes. Additionally, the samples were run on SDS gels to exclude proteolysis.

Elution volumes were converted into radii R via universal calibration at high ionic strength as outlined previously [18]. These radii are composed of two contributions, namely the hydrodynamic radius as a measure of shape in solution and a second term due to interfacial phenomena [18]. The hydrodynamic radius effective in GPC is very similar or even identical to a conventional viscosity radius (see Discussion).

Cross-linking with ethyleneglycolbis(succinimidylsuccinate)

GPC of rod and protofilaments was performed in 10 mM triethanolamine/HCl pH 8.0. The two rod peaks (dimers and tetramers, see Results) were collected. To avoid cross contamination only the trailing and leading edges were used for crosslinking experiments. Ethyleneglycolbis(succinimidylsuccinate) was added from a stock solution in dimethyl sulfoxide to a final concentration of 0.5 mg/ml. The proteins were present at a concentration of 50 µg/ml. After 15 min at 20 °C, the crosslinked products were precipitated by trichloroacetic acid, washed with acetone and analyzed by SDS gel electrophoresis using 5% polyacrylamide and a phosphate buffer. Control samples in which the crosslinker was omitted were treated in the same manner.

Quasielastic light scattering

Stokes radii were obtained from quasielastic light scattering experiments with GPC-purified samples as described previously [17].

Sedimentation analysis

The rod dimer and tetramer species as well as protofilaments were isolated by gel chromatography in 5 mM Tris/HCl pH 8.0. About 400 µl of the peak fractions were collected and sedimented in a Beckman model E ultracentrifuge at 20 °C. Protein concentrations ranged between 30–80 µg/ml.

Hydrodynamic calculations

Viscosity radii were determined from the radii measured by GPC on the calibrated TSK 5000 PW column by extrapolating the data to high ionic strength which eliminates contributions from interfacial phenomena. Hydrodynamic calculations were based on the experimental data obtained for molecular mass, sedimentation coefficient, Stokes radius and viscosity radius. Calculations were performed with a BASIC computer program, written to solve the iterative equations pertinent to stiff rods and allowing full flexibility in respect to the data set (molecular mass, sedimentation coefficient, Stokes radius, viscosity radius, hydration, partial specific volume and derived values such as intrinsic viscosity, frictional ratio, Scheraga-Mandelkern parameter, diameter and full length). The equations are discussed in [17].

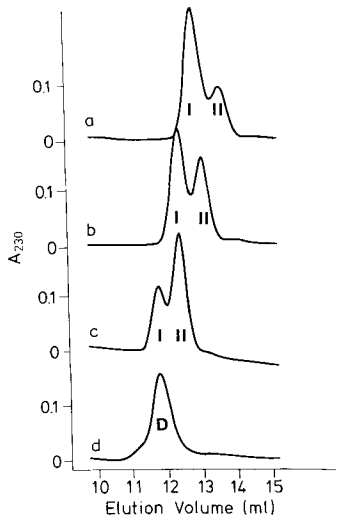

Fig. 1. *Elution profiles of desmin rod (a–c) and intact desmin (d) on a TSK G5000 PW gel permeation chromatography column.* Tris/HCl pH 8.0 of varying ionic strengths was used: 10 mM (I = 6 mM) in (a), 7.5 mM (I = 4.5 mM) in (b), and 5 mM (I = 3 mM) in (c and d). I and II mark the rod tetramers and dimers respectively. (d) The tetramic desmin protofilaments (D). Note that with decreasing ionic strength (a–c) the elution positions of identical protein complexes are shifted toward smaller elution volumes (see Results)

Electron microscopy

Rod dimers and tetramers were isolated as described above in 5 mM ethanolamine/HCl pH 8.0. Leading or trailing edges of the two peaks were collected, mixed with equal amounts of glycerol and sprayed onto freshly cleaved mica. Shadowing was performed with platinum/carbon at a fixed angle of 9°. Micrographs were taken at an original magnification of 37 500. The magnification was calibrated with catalase crystals as standard.

RESULTS

Gel permeation chromatography

Desmin rod was gel-filtered on TSK 5000 PW columns in buffered solution (pH 8) at various ionic strengths ranging from 0.17 M NaCl to plain 3 mM Tris/HCl (Fig. 1). Between the highest ionic strength conditions and about 20 mM Tris/HCl (I = 12 mM), the rod eluted as a single symmetrical peak. Upon further decrease in the ionic strength a second peak became apparent. It eluted closely after the first peak. With decreasing ionic strength, the relative amount of the second peak increased while the first peak decreased correspondingly. At 5 mM Tris/HCl (I = 3 mM) the second peak represented about 80% of the total absorbance but the exact value varied

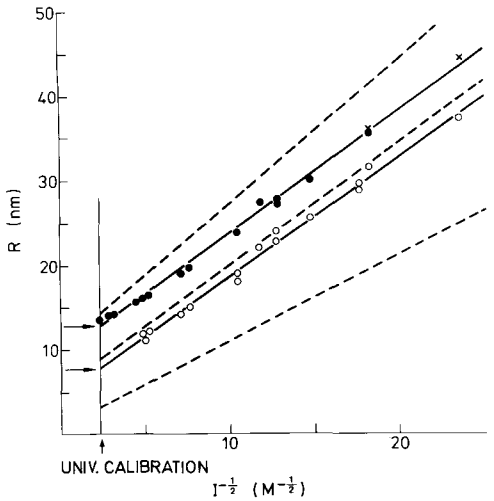

Fig. 2. *Ionic-strength dependence of GPC elution on a TSK 5000 PW column.* Desmin protofilaments (×), rod tetramer (●) and rod dimer (○) in ≤ 10 mM Tris buffer pH 8.0 plus NaCl to adjust to the desired ionic strength. The elution of ovalbumin (lower dotted line), thyroglobulin (middle dotted line) and bacteriophage MS2 (upper dotted line) are shown for comparison. Individual data for the latter proteins are given in Fig. 4 of [18]. All data were measured in a randomized sequence over a brief period of time to minimize changes of calibration as the column ages. Univ. calibration means universal calibration. The extrapolated viscosity radii are $R_n = 12.8$ nm for the rod tetramers and the protofilaments and $R_n = 7.7$ nm for the rod dimers

with the concentration of the rod solution applied to the column. Intact desmin did not pass through the column at ionic strengths higher than 5 mM Tris/HCl ($I = 3$ mM). In the latter buffer it eluted as a fairly symmetrical peak at the position observed for the first peak of the rod, but frequently a leading shoulder of less than 10% was present. However, contrary to the rod, which always eluted in high yield from the column, desmin was frequently only recovered at about 10–20% of the applied amount. SDS/PAGE demonstrated that the eluted material was intact desmin.

Fig. 1 shows also that the elution positions of the two peaks of the rod shift strongly upon decreasing the ionic strength. Thus at very low ionic strength (5 mM; Fig. 1c) the second peak elutes earlier than the first peak observed at high ionic strength (10 mM; Fig. 1a). Fig. 2 shows that the only peak observed at high ionic strength and the first peak observed at low ionic strength describe the same molecular species. Based on the sedimentation coefficient of the material present in the two peaks (I and II), the two species will be identified below as tetramers and dimers, respectively. Fig. 2 was obtained by converting the elution volumes measured at all ionic strengths into viscosity radii via the universal calibration obtained at high ionic strength (for a detailed description see [18]). Thus the apparent increase in size of the rod tetramer and dimer species with decreasing ionic strength follows a similar apparent increase observed with ovalbumin, thyroglobulin and MS2 phage. The effect of ionic strength on the GPC elution of conformationally unchanged molecules has been observed previously and seems to be due to interfacial phenomena. A physical interpretation, summarized in the picture of a counter ion cage that surrounds the macromolecule as a diffuse double layer, is thoroughly discussed elsewhere [18]. The detailed debate of these phenomena is however not crucial for the pursuit of the present hydrodynamic study, since interfacial contributions are cancelled by extrapolating the experimental radii to a high ionic strength [18]. From Fig. 2 we thus obtain a viscosity radius (R_n) of 12.8 nm for the rod tetramer and the protofilaments, while the rod dimer has a value of 7.7 nm. It is interesting to note, however, that the extent of the ionic strength dependency largely follows that observed for the globular reference proteins. Judged by the known net charge of desmin, desmin rod and reference compounds, conditions of saturated size of the diffuse double layer prevail (discussed in detail in [18]) and charge differences should not significantly influence the slopes under the chosen experimental conditions. It thus appears that the shape influences the ionic strength dependency very little but we cannot exclude that flexibility changes upon ionic strength alteration. As discussed [18], such changes are well documented for DNA and other systems, albeit the extent is generally much smaller that the additional interfacial effect observed in GPC.

Sedimentation of protofilaments and rod

Protofilaments and the two rod species were isolated by GPC in 5 mM Tris/HCl pH 8.0. At 50 μg/ml the rod dimer sedimented at $s_{20,w} = 2.85$ S, and the rod tetramer at $s_{20,w} = 3.85$ S. Rod dimer and tetramer species were rechromatographed after the sedimentation analysis was done. The dimer species seemed to be stable for several hours at room temperature while the tetramer species showed a slow conversion into dimers. Thus approximately 10% dimer was developed in at most 5 h from the previously isolated tetramer. Protofilaments sedimented at $s_{20,w} = 4.65$ S, but samples contained a faster migrating species of up to 20% of the total protein, sedimenting at approximately $s_{20,w} = 6.0$ S. Rechromatography of the sedimented sample did not however reveal any peak in addition to the original one. Rod tetramers were isolated by GPC in 40 mM borate pH 8.0, containing 98 mM NaF ($I = 100$ mM) and measured by quasielastic light scattering at 130 μg/ml. The Stokes radius was found to be $R_s = 9.3$ nm.

Hydrodynamic analysis of the molecules shape

We first used sedimentation coefficients and Stokes radii without reference to viscosity radii from GPC to calculate the molecular shape of rod tetramers as outlined in Materials and Methods. The results are calculated with a partial specific volume of 0.736 ml/g, the chemical molecular masses given by the known sequences [4, 14] of chicken desmin and its rod fragment with a hydration of 0.25 g/g. For the validity of the hydration value see [17]. The hydrodynamic length obtained for the rod tetramer, approximately 70 nm, clearly indicates that the dimers within the tetramers are staggered. We subsequently included viscosity radii in our calculation. The results for rod dimers and tetramers and the protofilament are summarized in Table 1. Any bias in the values of the viscosity radii (see Discussion) could affect the absolute numbers for the calculated length but hardly the length differences amongst the various species studied. However even the absolute values match those obtained by well tested absolute methodologies. Table 1 shows that protofilaments and rod tetramers exhibit the same hydrodynamic length (70 ± 5 nm) in line with their coelution from GPC.

Crosslinking results

Gel filtration in 10 mM triethanolamine/HCl pH 8.0 yielded, similar to the chromatography in Tris/HCl, the two

Table 1. *Hydrodynamic data*
The values for the diameters and the lengths were calculated from the experimental data set given on the left. For details see Methods and Results. The Stokes radius (R_s) was obtained from quasielectric light scattering experiments and the relative molecular masses from the sequence

Sample	M_r	$s_{20,w}$	R_n	R_s	Average diameter	Average length (± 5 nm)
		S	nm			
Desmin protofilament	212000	4.65	12.8		2.8	70
Rod tetramer	160000	3.85	12.8	9.3	2.5	71
Rod dimer	80000	2.85	7.7		2.3	40

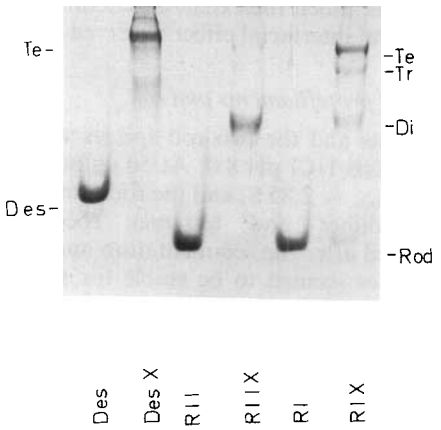

Fig. 3. *SDS/PAGE analysis of the crosslinked species formed by the peaks obtained from the TSK 5000 PW column.* The chromatography was as in Fig. 1, except for the buffer which was 10 mM triethanolamine/bicarbonate pH 8.0. Under these conditions rod dimers and tetramers were present in nearly equal amounts and desmin protofilaments eluted at the position of the rod tetramer. The samples from the desmin peak (Des), the rod dimer peak (RII) and the rod tetramer peak (RI) (compare Fig. 1) are displayed in pairs before and after crosslinking: Te stands for tetramers, Tri for trimers and Di for dimers. Note the high yield of tetramer species from the rod tetramer peak (RIX) and the formation of dimer species from the rod dimer peak (RIIX) (see Results)

rod species in approximately equal amounts and a single peak of protofilaments, which eluted in the position of the tetrameric rod species. Crosslinking of the three species was performed under identical conditions (protein concentrations, GPC solvent, temperature and time). The analysis of the crosslinked probes by SDS/PAGE (Fig. 3) revealed the nearly exclusive formation of tetramers in the case of protofilaments (Fig. 3, slot Des X) and a distinct difference between the two rod peaks. The rod tetramer peak (Fig. 3, slot RI) provided primarily crosslinked tetramers and only some dimer and trimer forms. In contrast, the rod dimer peak (Fig. 3, slot RII) showed primarily dimers with only trace amounts of tetramers visible.

Electron microscopy

For electron microscopy the two rod species were isolated by GPC in 5 mM ethanolamine/HCl pH 8.0. Among a variety of buffers tested, this solvent seems to provide in our hands the clearest micrographs. The rod tetramer molecules revealed a length distribution of 65 ± 5 nm, while the dimer peak showed molecules with a length of 50 ± 5 nm (Fig. 4). The dimers seemed somewhat thinner that the tetramers, which frequently displayed bends most likely corresponding to extending single coiled-coil regions (see Discussion).

DISCUSSION

The organization of the protofilament is a central problem for the understanding of the overall structure of the intermediate filament. It seems now firmly established that the building block is a tetramer. Neglecting the short non-helical domains at the ends, the tetramer is a dimer of a double-stranded coiled coil. We have approached the problem of the relative orientation of the two coiled coils by a proteolytically derived fragment (residues 70–415), the rod, which spans the entire central α-helical portion of the molecule and has lost the non-helical head and tail domains [4]. As this fragment lacks the ability to polymerize, it is particularly suitable for hydrodynamic studies at moderate ionic strength. In buffers of increased to moderate ionic strength, the rod elutes in gel filtration as a single peak. When the ionic strength is decreased below approximately 50 mM salt, a second later-eluting peak becomes apparent. This peak increases in relative amount upon further decrease in ionic strength (Figs 1, 2). Molecular characterization by sedimentation and by chemical crosslinking (Fig. 3), shows that the first peak contains tetramers (two coiled coils) and the second peak dimers (single coiled coils). The separation of the two species by GPC is good enough to yield pure preparations, which appear to be relatively stable, i.e. they convert into each other only relatively slowly.

The universal calibration of the GPC column at high ionic strength in combination with extensive filtration series in buffers of increasingly lower ionic strength [18] allowed the determination of the viscosity radii for species that only exist at lower ionic strength, although the elution positions of identical species shifted considerably with the ionic strength. It has been demonstrated previously that all polyelectrolytes exhibit this ionic-strength-dependent shift of retention volumes, independent of aggregation or conformational changes such as denaturation [18]. This observation has been reported by a variety of investigators (reviewed in [18] which also proposes a novel physical interpretation in terms of interfacial phenomena). The ionic strength dependency, whatever its detailed physical origin may be, must be cancelled by extrapolation to the high ionic strength used for universal calibration in order to obtain hydrodynamic shape information from GPS. Together with the additionally determined sedimentation velocity values, the Stokes radius from quasielastic light scatter-

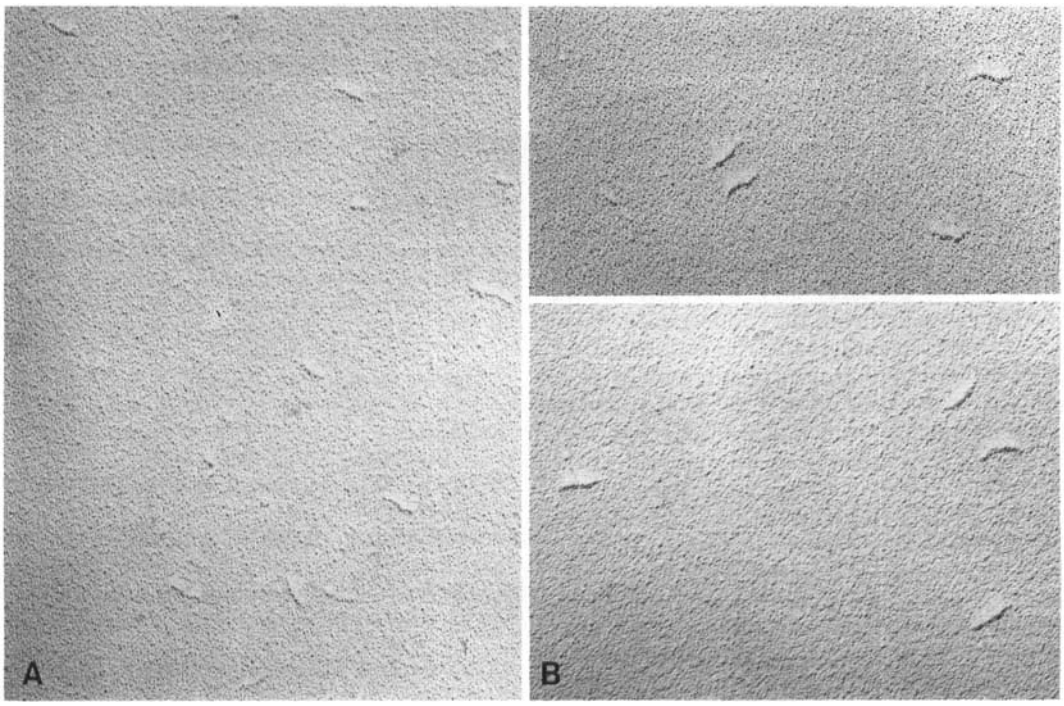

Fig. 4. *Electron micrographs of rod dimers (A) and rod tetramers (B).* Isolation of the two species was achieved by GPC in 5 mM ethanolamine/bicarbonate buffer pH 8.0. Molecules were shadowed with platinum/carbon at a fixed angle of 9°. Dimers measure 50 ± 5 nm in length, and tetramers 65 ± nm ($n = 100$). The magnification is 80000-fold in both A and B

ing and the known molecular masses, the shape of the two species could be calculated. The hydrodynamic length values derived for the dimer and tetramer are 40 ± 5 and 71 ± 5 nm respectively and agree with values exclusively based on conventional hydrodynamic methodologies (Table 1). A recent report generally confirms the concept of universal calibration in terms of viscosity radii by excluding Stokes radius, radius of gyration, mean linear extension and other proposals but claims a systematic basis for stiff rods to elute slightly earlier than predicted by intrinsic viscosity. This deviation supposedly increases with increasing persistence length [19]. Since we obtained independently a Stokes radius which confirms the hydrodynamic length of 70 nm, this deviation only suggests that our particles may be more flexible than anticipated. These estimates are in line with the electron microscopic appearance of the isolated dimers and tetramers. Here the corresponding length measurements center around 50 nm and 65 nm, respectively (Fig. 4).

The protofilament unit of the complete protein, i.e. the rod domain plus the terminal head and tail domains, elutes from the GPC column at the same position as the rod tetramer when most highly diluted buffers are used (3 and 5 mM Tris/HCl; Figs 1, 2). By hydrodynamic analysis and chemical crosslinking this species is clearly defined as tetramer. Notably most of the extra mass is reflected in $s_{20,w}$ (4.65 S vs 3.85 S), whereas the viscosity radius remains unchanged (12.8 nm). By inspection this indicates that both structures must have similar lengths while thickness increases. Indeed a detailed hydrodynamic calculation confirmes that protofilaments and the rod tetramer have the same lengths (Table 1).

The experimentally derived length of the rod dimer, i.e. the single coiled coil, is between 40 nm (hydrodynamic calculation) and 50 nm (electron microscopy), which in good agreement with the length expected for an extended α-helix with approximately 280 residues in α-helical conformation (see the predictions from the sequence in [4]). A similar length for a single coiled coil has been obtained for desmin dimers in 3 M guanidine hydrochloride (45 nm) [20]. Thus the length of the rod tetramer (65 – 70 nm) predicts that its two coiled coils are staggered by about 15 – 30 nm. They therefore overlap by about 25 – 35 nm. These values are in good agreement with a recent electron microscopic analysis of paracrystals formed by the rod domain of glial fibrillary acidic protein [21, 22]. This study deduces the length of the single coiled coil to 48 nm. The overlapping part between the coiled coils is given as 34 nm and the stagger as approximately 14 nm in length. In addition the crystals indicate antiparallel arrangement of the two coiled coils with the N-terminal parts present in the inner overlapping region. Thus the extending parts are formed by the carboxy-termini of the rod [22]. Based on the labeling of the desmin rod with Fab fragments derived from a monoclonal antibody directed against the carboxy-terminal part of the rod domain, we have previously proposed a similar antiparallel arrangement [9]. Although in this earlier study we assumed the tetramer to be fully overlapping at a total length of 48 nm, the labelling pattern observed would also conform with a tetramer displaying a 14-nm stagger (outlined in detail in [22]). On the basis of our present analysis, we have to conclude that previous electron microscopic studies by us and others did not consider the possibility of a tetramer – dimer dissociation. Thus the 48-nm-long rodlets observed originally by us in the case of desmin rod preparation [11] were most likely dimers as the material was processed in 2 mM Tris/HCl pH 8.0. The staggered and antiparallel form of the tetramer also seems to be in agreement with the biochemical analysis of tryptic fragments of keratin [5, 23] and desmin filaments [24]. Here the N-terminal part of the rods were characterized as tetramers, while the C-terminal parts were clearly dimers. In the case of desmin this dimer was shown to cover residues 317 – 406. The calculated as well as experimentally derived

length given as 13 nm [24] coincides nicely with the stagger of 14 nm now directly observed on paracrystals of the rod of glial fibrillary acidic protein [22].

Although our results on desmin protofilaments are not complete, they suggest that at least those molecules which are recovered from GPC reflect the arrangement of the rod tetramer. Nevertheless even in dilute buffers these protofilaments remain largely as tetramers. We assume that this is the consequence of the presence of the N-terminal head domain (residues 1–69; see also [10]). The C-terminal tail domain (residues 416–463) probably adds very little to the protofilament length compared with the rod tetramer. Based on earlier experience with vimentin [14], it is possible that, for this protein, dimer formation of protofilaments may be more easily achieved than in the case of desmin. When analyzed by GPC, the leading fraction of the peak had primarily molecules of 64 nm in length, while a minor fraction enriched at the trailing edge was characterized as 45 nm long. Nevertheless, since the fraction enriched in shorter molecules had not been characterized by sedimentation velocity or molecular mass, the assignment as dimers remains tentative.

In earlier electron microscopic studies the majority of desmin protofilaments have been characterized with a length of 53 or 48 nm [7, 13, 25]. At this time it remains open whether molecules of this short length reflect unstaggered tetramers, staggered tetramers with the extension folded back or indeed dimers.

Although the hydrodynamic characterization and the paracrystals begin to give a reasonable model for the tetramer of the rod, the antiparallelity invoked [11, 22] would make the filament an apolar structure while F-actin and microtubules are clearly polar organizations. Vectorial assembly of intermediate [26, 27] and the postulated anchorage at the nuclear lamina and in the subplasma membrane microfilament system could still be fulfilled by apolar intermediate filament structures since the two 'receptors' (lamin B, ankyrin) are present in different compartments. Nevertheless, given the tendency of intermediate filament to polymorphism, a difference between *in vitro* and *in vivo* assembly cannot be dismissed. However the beginning of an understanding of the complexes formed *in vitro* will surely lead to a better characterization of the formation of intermediate filaments.

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