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Flow birefringence property of desmin filaments

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We have investigated the flow birefringence property and assembly process of desmin, a muscle specific intermediate protein. Solution of non-polar desmin filaments showed birefringence when aligned in the sheared flow. The amount of birefringence of desmin filaments was considerably lower when compared with that of F-actin solution. Assembly of desmin from soluble state was followed by the birefringence measurements. At any desmin concentrations examined, the degree of flow birefringence increased rapidly just after the addition of the assembly buffer and reached a saturated level within 30 min. The time to reach half-maximal values of flow birefringence slightly but definitely depended on the initial soluble desmin concentrations. The plotting of the initial velocity of the assembly against the soluble desmin concentrations showed a slope of 1.4. This result suggested that the assembly process detected by flow birefringence measurements followed second-order kinetics, and the process corresponded to the second step of the three stage model for type III intermediate filament assembly proposed by Herrmann and his colleagues; the annealing of unit length filaments into filaments.

Key words: desmin, intermediate filament, flow birefringence

Intermediate filaments (IFs) with diameters of 10–12 nm are stress-bearing cytoskeletal elements of eukaryotic cells and play critical roles in formation of dynamic cell architecture (for recent reviews, refer[1,2,3]). Recent studies have revealed that the molecular architecture and assembly mechanism of IFs follow quite different rules from those of polar microfilaments and microtubules in which globular subunits are arranged in helical symmetry[4,5].

IFs are non-polar filaments without discrimination of head and tail on their filaments ends. Building units of IFs are parallel rod-shaped dimers which are stabilized through α-helical coiled-coil interactions of the polypeptides[6,7,8]. The coiled-coil stretched over 310 amino acids in the middle of IF proteins and is called as the rod-domain. The N-terminal containing head and C-terminal containing tail domains flank the rod domain. The three domain structures are common to more than a hundred of isomer subunits of IFs[7,9,10]. These IF subunit proteins are classified into five sequence homology types; acidic and neutral-basic keratins are grouped into type I and II, respectively, type III contains desmin, vimentin and glial acidic fibrillar protein (GAFP), subunits of neurofilamets constitute type IV, and nuclear lamins are classified as type V[7,11,12].

Polymerization-depolymerization cycle of actin and tubulin can be repeated *in vitro* by changing their solvent conditions. On the other hand, high concentrations of denaturing reagents such as 6 M urea are required for the depolymerization of purified IFs *in vitro*[13,14]. After dialysis against a low ionic strength buffer at slightly alkaline pH, the soluble fraction is obtained[14,15]. The major component of this soluble fraction is tetramers. The tetramers are apolar structure because they consist of two dimer molecules aligned in antiparallel and half-staggered manner[16,17,18]. This soluble fraction has been used as a starting material for the assembly experiments *in vitro*. Assembly *in vitro* can be induced by increasing ionic strength and lowering the pH to neutrality[13,15]. The time courses of the assembly of IFs are shown to be very quick process. Results from viscometry or turbidometry of the assembly indicated that half-maximal values of filament formation reached within 5 min and a plateau

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within 30 min[19] after the addition of salt. This quick initial reaction makes the analysis of IF assembly process by ordinal kinetical techniques difficult[12].

By applying quantitative approaches to electron microscopy, Herrmann and his colleagues have investigated the assembly mechanism of the type III subunits in detail[3,20,21]. They have documented the assembly process in three-step scheme; 1) formation of unit length filaments (ULFs) of 60 nm long and 15 nm in diameter, 2) sequential association of ULF complexes to form immature IFs with diameters larger than mature IFs, and 3) radial compaction of the immature IFs into the mature IFs.

The ULF particles, formed by the lateral association of eight tetramers[16,17], are apolar structure because tetramers are apolar structure. End-to-end annealing of tetramers explains the formation of filaments without polarity. They showed that the most critical step of the assembly, ULF formation, is completed within seconds after the addition of the assembly buffer.

Assembly of IF proteins are regulated by solvent conditions, modification of proteins[22,23], and various regulatory proteins[24]. In order to elucidate the molecular mechanism of IF assembly and their regulation process, a wide range of physico-chemical techniques have been applied[1,2,3,21].

Flow birefringence measurement is a useful technique for analyzing filamentous molecules in solution[25,26]. In this report, we examined the flow birefringence properties of desmin filaments and their assembly properties.

Materials and Methods

Preparation of desmin

Desmin was prepared from frozen chicken gizzards purchased from a commercial market by successive extraction with 0.6 M KCl solution and 0.6 M KI solution as described by Geisler and Weber[14]. Desmin in the KI-insoluble residues were solubilized with 6 M urea solution (6 M urea, 1 mM EGTA, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 25 mM Tris-HCl, pH 7.5). Purified desmin was obtained by DEAE column chromatography[13]. DE-52 (Whatman) column equilibrated with 6.5 M urea, 1 mM EGTA, 0.5 mM PMSF, 0.5 mM DTT, and 25 mM Tris-HCl, pH 8.0 was eluted with a linear gradient of NaCl, and fractions eluted with 40–80 mM NaCl were collected. After concentration with an Amicon Ultra-4 membrane (Milipore), purified desmin was stored at −80°C. Concentrations of desmin were spectrophotometrically determined using extinction coefficient[13] at 278 nm of 0.56 mg^{-1} ml cm^{-1}.

Assembly of desmin

Desmin prepared as describe above was dialyzed overnight at 4°C against 0.5 mM DTT and 5 mM Tris-HCl, pH 8.0 with frequent changes of the dialysis buffer. After clarification for 15 min at 10,000×g, the supernatant (called as soluble desmin in this paper) was used for experiments.

Assembly of soluble desmin was initiated by the addition 1/10 volume of assembly buffer to give the final solvent condition of 0.15 M NaCl and 25 mM Tris-HCl, pH 7.2.

Flow birefringence measurements

The flow birefringence and the extinction angle (χ) were measured using a Micro FBR mark II apparatus (Wakenyaku) with a cell of 1 cm in optical path at room temperature. For convenience, the flow birefringence was expressed as in degree ($\Delta°$).

Viscosity measurements

Viscosity was measured with Ostwald-type viscometers (Shibata) with sample volume of 0.5 ml and buffer flow times around 60 sec at 25.0°C.

Other physico-chemical techniques

SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli[27] using 10% separating gel and protein bands were detected by Coomassie Brilliant Blue. Negative staining was carried out with 2% uranyl acetate and electron microscopy was done on a JEM 100C electron microscope at an accelerating voltage of 80 KV

Results and Discussion

Desmin with a subunit molecular weight of 53 k is muscle-specific IF protein that encircles Z-disks of sarcomeres and keeps the parallel array of myofibrils in registered[28,29]. The SDS-polyacrylamide-gel electrophoresis pattern of the desmin preparation used for the present experiments was shown in Figure 1a. The preparation contained a small amount of degradation products but did not contain any other impurities. Electron microscopy of negatively stained desmin filaments showed smooth filaments of various lengths with an average diameter of 11 nm (Fig. 1b). Negatively contrasted images of the soluble desmin fraction revealed rod-like particles of average lengths of 59.0±12.5 nm when 98 particles were measured (Fig. 1c). The observed size corresponded with the particle length of soluble subunits reported by Hermann *et al.*[16].

Flow birefringence property of desmin filaments

Soluble desmin was assembled by the addition of assemble buffer and stood overnight at room temperature. Degree of flow birefringence and extinction angels (χ) of the desmin filaments were measured at various velocity gradients (Fig. 2). The degree of birefringence ($\Delta°$) increased as the velocity gradients (G) increased. The observation indicated that desmin filaments aligned by the flow. Overall profiles of the plots were similar to those reported for F-actin[25] showing that polymerized desmin solution contained long filaments of heterogeneous lengths. As long as we have examined so far, the plots of extinction angels (χ) against the velocity gradient (G) were not extrapolated to 45° at G=0. This

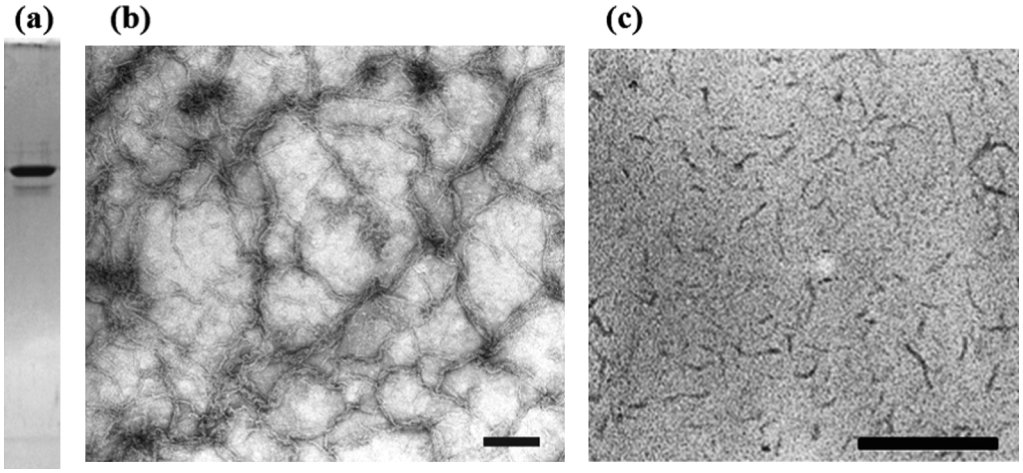

Figure 1 SDS-polyacrylamide gel electrophoresis pattern of the desmin preparation and negatively stained images of soluble and assembled desmin. (a) SDS-gel pattern of desmin preparation used. Negatively stained image of desmin filaments assembled in 0.15 M NaCl and 25 mM Tris-HCl, pH 7.2 (b) and soluble desmin in 0.5 mM DTT and 5 mM Tris-HCl, pH 8.0 (c). Bar indicates 200 nm.

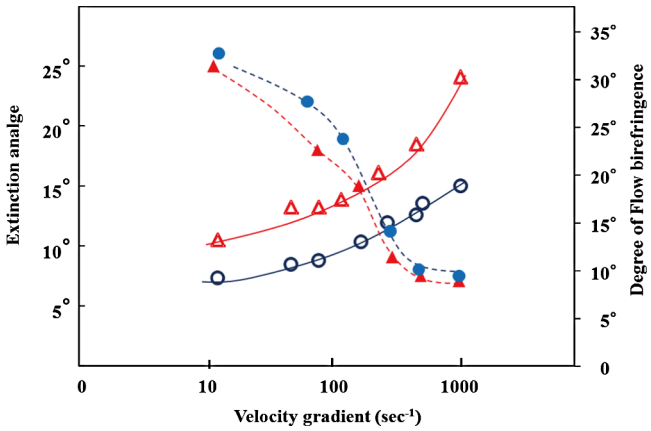

Figure 2 Flow birefringence properties of desmin filaments. The amounts of flow birefringence and extinction angles were measured at various velocity gradients. Desmin concentrations, 1.24 mg/ml (circles) and 1.64 mg/ml (triangles). Open symbols, flow birefringence. Filled symbols, extinction angles.

observation suggested the presence of molecular interaction between desmin filaments especially at low shear rates. Assuming the axial ratio of the filament to be 100, the length of desmin filament was calculated from the value of extinction angle[30,31]. At G=12 sec^{-1}, apparent particle length was calculated to be 2.5 and 2.1 μm for 1.24 mg/ml and 1.64 mg/ml desmin filaments, respectively. At higher velocity gradient (1000 sec^{-1}), apparent length was 1.5 and 1.2 μm for 1.24 mg/ml and 1.64 mg/ml desmin filaments. There are limited numbers of reports on the length distribution of IFs in vitro. From electron microscopy of negatively stained images, Angelides *et al.*[32] reported average length of NF-L (class IV) to be 0.5±0.1 μm. Atomic force microscopy of desmin filaments by Kiss *et al.* showed average length of 0.8 μm[33].

When compared with F-actin solution, the flow birefringence of desmin filaments was considerably small. The degree of flow birefringence of 1 mg/ml F-actin solution at G=1000 sec^{-1} was 90° and the birefringence (Δn) was calculated to be 1.35×10^{-7}. On the other hand, the degree of flow birefringence of 1 mg/ml desmin filaments was 15.5° (Δn, 1.6×10^{-8}). It is possible that the non-polar nature of desmin filaments contributed this low birefringence.

Critical concentrations at equilibrium

Degrees of flow birefringence of desmin filaments at equilibrium were plotted against soluble desmin concentrations (Fig. 3). The amount of birefringence increased linearly with the soluble desmin concentrations. Extrapolation of the plots intercepted the abscissa at the desmin concentration of 0.05 mg/ml. Similar value (0.08 mg/ml) was obtained from the measurements of specific viscosity of desmin filaments. These values showed good agreement with the reported critical concentrations of desmin assembly determined by turbidometry and centrifugation assay[34]. It is to be noted that the exchange of IF subunits in living cells takes place not at filament ends but alongside the filaments[32,35].

Time course of desmin assembly

Herrmann and his colleagues have shown that the assembly of IFs from the soluble subunits is very quick process[3,20,21]. Measurements of the time courses of the assembly by viscometry or turbidometry showed that the time required to attain half-maximum values of the filament formation was not affected by soluble subunit concentrations[19,21].

We followed the time course of desmin assembly by flow birefringence measurements. The soluble desmin consisted of particles of ~60 nm long as a major component (refer Figure 1c), but flow birefringence of the soluble desmin

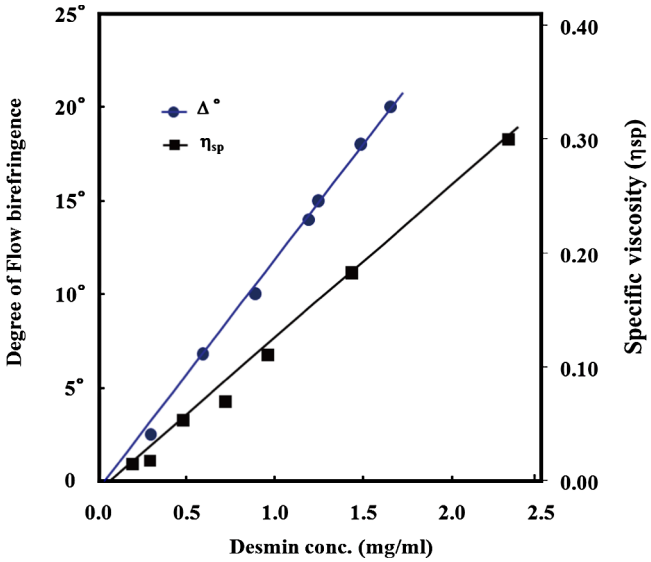

Figure 3 Relationship between desmin concentrations and the steady-state values of desmin filaments determined by flow birefringence and viscosity measurements. The degree of flow birefringence and specific viscosity (η_{sp}) of desmin filaments obtained after overnight assembly were plotted against soluble desmin concentrations. Measurements of viscosity were carried out at 25.0°C. Degree of flow birefringence (Δ°) at a velocity gradient of 500 sec^{-1} were measured at room temperature. Solvent conditions, 0.15 M NaCl and 25 mM Tris-HCl, pH 7.2.

solution was very low. The degree of flow birefringence of 2.0 mg/ml soluble desmin at a velocity gradient of 1000 sec^{-1} was 4°. The transient electric birefringence measurements done by Koojiman *et al.*[36] indicated that soluble vimentin and GAFP (type III isomers) showed the permanent dipole and the dipole quickly disappeared after the addition of salt. On the other hand, flow birefringence of desmin increased quickly just after the initiation of the assembly.

In order to trace the quick initial assembly process, the assembly buffer was added to the soluble desmin in the cell of the birefringence apparatus, and the mixing was done by applying the shear for measurement (500 sec^{-1}). At any desmin concentrations examined, the degree of flow birefringence increased rapidly just after the addition of the assembly buffer and reached a saturated level within 30 min (Fig. 4a). The time to reach half-maximal values of the flow birefringence slightly but definitely depended on the initial soluble desmin concentrations. They were 105, 74, 50, and 25 sec for 0.42, 0.83, 1.24, and 1.64 mg/ml desmin. The plots of the initial velocity of the assembly against the soluble desmin concentration showed a slope of 1.4 (Fig. 4b). This result suggested that the assembly process detected by the flow birefringence measurements followed second-order kinetics, and the process corresponded to the second step of the three stage model, the annealing of ULFs into filaments, proposed by Herrmann and his colleagues[18,19].

Conclusion

Solution of non-polar desmin filaments showed birefringence when aligned in the sheared flow. The amount of birefringence was considerably lower when compared with that of F-actin solution. We considered that non-polar structure of IFs partially contributed to the low birefringence. On the other hand, flow birefringence measurement was useful to analyze assembly process of intermediate filaments. Our results suggested that rapid increase of birefringence

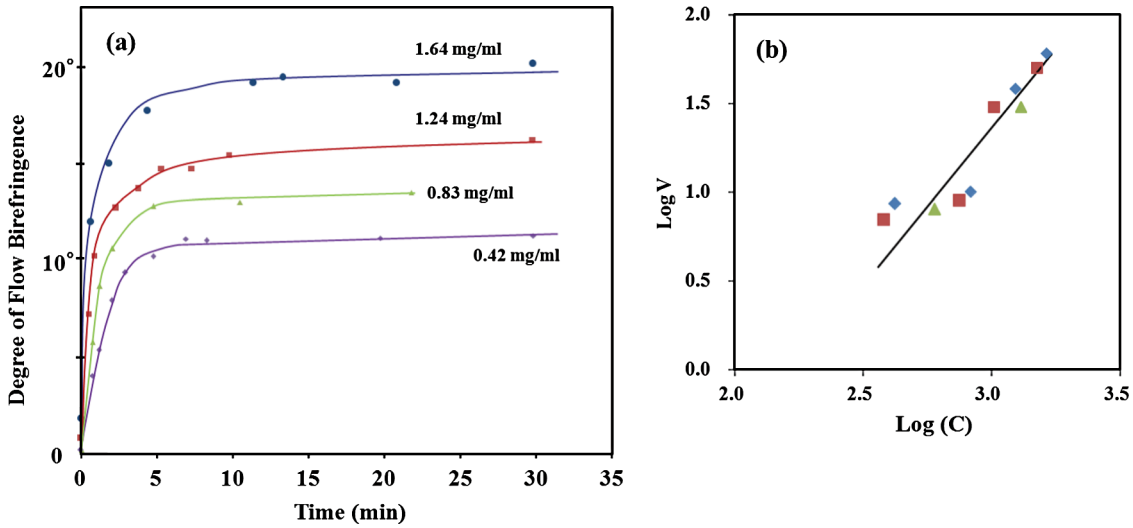

Figure 4 Time course of desmin assembly measured by flow birefringence. Assembly of the soluble desmin was initiated by the addition of one-tenth volumes of assembly buffer. Measurements of the degree of flow birefringence at a constant velocity gradient of 500 sec^{-1} were carried out at room temperature. (a) Time course of desmin assembly. Solvent conditions, 0.15 M NaCl and 25 mM Tris-HCl, pH 7.2. (b) Relationship between the desmin concentrations and the initial velocities of the assembly. Abscissa, logarithm of desmin concentrations in μg/ml. Ordinate, logarithm of initial velocity of assembly (degree/min). Different symbols indicate different measurements.

detected at the early stage of desmin assembly corresponded end-to-end reaction of ULFs.

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