Determination of the critical concentration required for desmin assembly

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The critical concentration required for filament assembly *in vitro* from highly purified desmin was determined by both turbidity and centrifugation assays. Assembly was done in the presence of 2 mM- Ca^{2+} , 2 mM- Mg^{2+} or 150 mM- Na^{+} at 2, 22 and 37 °C. Similar values for critical concentration were obtained by both assays. As temperature increased, critical concentration decreased for each cation. The critical concentration was lowest in the presence of Ca^{2+} at 2, 22 and 37 °C, but was highest in the presence of 150 mM- Na^{+} at 2 °C. Negative staining showed that supernatants from the centrifugation assays contained protofilaments, protofibrils and short particles (< 300 nm), but pellets contained long filaments (> 1 μm) with an average diameter of 10 nm. As the temperature increased, both the average diameter and average length of particles in the supernatant increased. Thermodynamic analysis indicated that hydrophobic interactions were dominant during desmin assembly, but that ionic interactions might also be involved. Our results demonstrated that the specific cation and temperature and temperature-cation interactions all are important in assembly of desmin intermediate filaments.

INTRODUCTION

Intermediate filaments (IF), which are part of the cytoskeleton of most cells, average 10 nm in diameter and can be classified into six subclasses based on their origin: keratin (epithelial cells), vimentin (cells of mesenchymal origin), desmin (muscle cells), glial fibrillary acidic protein (GFAP) (glial cells), neurofilament proteins (neurons) and lamins (inner surface of the nuclear envelope). Recently, on the basis of comparisons among primary sequences, IF proteins have been grouped into five sequence types: Type I (acidic keratins); Type II (neutral/basic keratins); Type III (desmin, vimentin, GFAP); Type IV (neurofilaments: NF-L, NF-M, NF-H); and Type V (lamins A, B and C) (Steinert & Roop, 1988; Robson, 1989). Solubility of IF proteins in the filamentous state is relatively low, and their isolation and purification involve a denaturing solvent such as urea to solubilize the IF protein (Huiatt *et al.*, 1980; O'Shea *et al.*, 1981; Vorgias & Traub, 1983). Purified Type III IF proteins, however, will remain soluble without denaturing conditions if the ionic strength is kept low (< 0.01) and the pH is slightly alkaline. The properties of desmin and desmin IFs have recently been reviewed (Stromer, 1990).

It has been shown by various approaches that the basic building blocks of IF are protofilaments (Stromer *et al.*, 1981; Aebi *et al.*, 1983), which are tetramers composed of four monomers in the form of a pair of coiled-coils (Geisler & Weber, 1982; Pang *et al.*, 1983; Pang & Robson, 1984; Ip *et al.*, 1985b). Monomers of IF proteins share a common structure that contains a conserved, α -helix-rich rod domain and a non-helical *N*- and *C*-terminal (head and tail respectively) domains (Geisler & Weber, 1982; Robson, 1989). The *N*-terminal, but not the *C*-terminal, domain plays a functional role in filament formation (Traub & Vorgias, 1983, 1984; Kaufmann *et al.*, 1985; van den Heuvel *et al.*, 1987).

Purified Type III IF proteins, in general, are able to form filaments that are very similar to native IFs if the ionic strength is increased, if pH is decreased close to 7 or if both are done (Huiatt *et al.*, 1980). Assembly is also greatly influenced by specific cations and temperature. Previous studies from our

laboratory (Stromer *et al.*, 1987) have shown that, at the same ionic strength, bivalent cations are more effective promoters of desmin assembly than univalent cations, and that rate of assembly increases as temperature increases from 2 to 37 °C. Similar results on assembly of GFAP were obtained by Yang & Babitch (1988). There are, however, no published data on the critical concentration required for desmin assembly under a specific set of conditions. Knowing the critical concentration under different conditions has the potential to help us understand the mechanism and forces involved in assembly. The purpose of this study, therefore, was to determine the critical concentration required for desmin assembly in the presence of specific combinations of cations and temperature. Assembly was monitored by two independent methods, namely, turbidity and centrifugation, and was verified by negative staining of pellets and supernatants obtained during the centrifugation assay.

MATERIALS AND METHODS

Desmin preparation

Desmin was isolated and purified from fresh turkey gizzards by the method of Huiatt *et al.* (1980). Urea was removed from the purified desmin by extensive dialysis against 10 mM-Tris/acetate, pH 8.5, at 2 °C. Before desmin was used for assembly, it was dialysed overnight against two changes of 1 mM- NaHCO_3 , pH 8.0, at 2 °C and then clarified by centrifugation at 110 000 *g* (r_{av} , 59.1 cm) for 30 min. The desmin concentration in the supernatant was determined by spectrophotometry [$A_{278\text{ nm}}^{1\%} = 5.57$ (Huiatt *et al.*, 1980)]. This value is consistent with that reported for desmin by Steinert *et al.* (1981).

Assembly of desmin

Desmin assembly was initiated by adding 0.6 ml of filament-forming buffer (30 mM-imidazole/HCl, pH 7.0, plus the respective cation) to 1.2 ml of desmin. Final desmin concentrations tested were 100–350 $\mu\text{g/ml}$ for assembly at 2 °C, 50–250 $\mu\text{g/ml}$ at 22 °C and 25–125 $\mu\text{g/ml}$ at 37 °C. The final concentrations of cations were 2 mM- CaCl_2 , 2 mM- MgCl_2 or 150 mM- NaCl .

Assembly was monitored by measuring the turbidity at 300 nm when maximum absorbance was reached (16 h for 2 °C, 8 h for 22 °C and 3 h for 37 °C). Turbidity was corrected for absorbance due to unassembled desmin by using control solutions of desmin in 1 mM-NaHCO₃ and due to the assembly buffer by using blanks in which no desmin was present.

Another aliquot was centrifuged after maximum absorbance was reached to pellet desmin filaments and leave a constant amount of protein in the supernatant at different starting protein concentrations. Centrifugation was done either at 40 000 *g* (r_{av} , 59.1 cm) for 30 min for desmin assembly in the presence of 2 mM-CaCl₂ or -MgCl₂ at 2 and 22 °C or at 12 000 *g* (r_{av} , 59.1 cm) for 30 min for the other temperature-cation combinations. The centrifugal force selected was based on our experience with the length of filaments formed under a given set of conditions, the force required to sediment them and the requirement that the amount of desmin remaining in the supernatant must remain constant as the starting desmin concentration is increased. The desmin concentration in the supernatant was determined by the Bradford (1976) method, using desmin to determine the standard curve.

Negative staining

Aliquots from both pellets and supernatants were negatively stained for electron microscopy. A drop of the sample was added to a glow-discharged carbon-coated grid. After 3 min, each grid was washed with five drops of glass-distilled water, stained with 2% uranyl acetate, and then examined at 80 kV in a JEOL JEM-100CXII electron microscope. Images were recorded on Kodak SO-163 electron image film at a nominal magnification of $\times 40000$. Actual magnification was determined by photographing a carbon-grating replica (E. F. Fullam, Latham, NY, U.S.A.). A clear acetate sheet with lines 4 mm apart was randomly placed over the negative. Diameters and lengths of all recognizable particles that intersected a grid line, including protofilaments, protofibrils and filaments, were measured with a Bioquant Image Analysis System (R & M Biometrics, Nashville, TN, U.S.A.) by the method of Stromer *et al.* (1987).

RESULTS

Turbidity

Fig. 1 shows an average of four replications of a turbidity assay done at 37 °C with three cations. At 37 °C, the highest final turbidity was obtained with Ca²⁺, followed in decreasing order by the turbidities obtained with 2 mM-Mg²⁺ and with 150 mM-Na⁺. When identical experiments were done at 22 or 2 °C, the order for the final turbidities was 150 mM-Na⁺ > 2 mM-Ca²⁺ > 2 mM-Mg²⁺ (results not shown). These results indicate that there

Fig. 1. Desmin assembly at 37 °C in the presence (separately) of three cations

Desmin was assembled at 37 °C in 2 mM-CaCl₂ (□), in 2 mM-MgCl₂ (○) or in 150 mM-NaCl (△), each buffered with 10 mM-imidazole/HCl, pH 7.0. Each point is the average for four separate determinations. Values were corrected for desmin and buffer absorbance as described in the Materials and methods section.

is a temperature-cation interaction with Ca²⁺ at 37 °C that affects the turbidity of desmin particles in suspension, and confirms the previous observation of Stromer *et al.* (1987). For a given cation, turbidity is directly proportional to the desmin concentration. Critical concentration is determined by extrapolating an individual straight line for each cation to determine the intercept on the x-axis where the turbidity increment is equal to zero. The intercepts, therefore, represent the highest desmin concentrations with no detectable turbidity increase or assembly, i.e., the critical concentration. Fig. 1 indicates that critical concentration for desmin assembly at 37 °C in the presence of 2 mM-Ca²⁺ or 150 mM-Na⁺ is 11.6 μg/ml, but in the presence of 2 mM-Mg²⁺ is 15.4 μg/ml. In addition, for assembling a given total amount of desmin, considerably higher turbidity was observed in the presence of 2 mM-Ca²⁺ than in the presence of 2 mM-Mg²⁺ or 150 mM-Na⁺ at 37 °C (Fig. 1). The higher turbidity in the presence of Ca²⁺ involved formation of larger particles that are aggregates of filaments. These filamentous aggregates did not all remain suspended in the filament-forming buffer and gradually settled as a small amount of flocculent material at the bottom of the cuvette. This phenomenon also has been noted in our previous

Table 1. Critical concentration (μg/ml) of desmin determined by turbidity (T) and centrifugation (C) methods

Temperature (°C)	Critical concentration								
	2 mM-Ca ²⁺			2 mM-Mg ²⁺			150 mM-Na ⁺		
	T*	C*	$\bar{x}$ †	T	C	$\bar{x}$	T	C	$\bar{x}$
2	30.4 ± 6.6	29.1 ± 3.6	29.8 ± 7.8	36.7 ± 5.3	31.5 ± 5.0	34.1 ± 7.5	55.5 ± 8.1	51.8 ± 2.8	53.7 ± 9.8
22	20.0 ± 5.5	17.6 ± 2.1	18.8 ± 6.9	26.2 ± 4.7	25.2 ± 2.7	25.7 ± 5.4	28.2 ± 2.5	20.4 ± 1.3	24.3 ± 3.7
37	11.6 ± 2.0	3.7 ± 0.4	7.7 ± 3.2	15.4 ± 2.8	14.0 ± 1.8	14.7 ± 4.3	11.6 ± 2.9	8.5 ± 0.7	10.1 ± 3.1
Overall average...			18.8 ± 9.7			24.8 ± 10.3			29.4 ± 15.7

* Each mean value with its S.E.M. is calculated from four replications with duplicate samples.

† Average of values determined from turbidity and centrifugation methods.

Fig. 2. Supernatant desmin concentration after centrifugation of desmin assembled at three temperatures in the presence of 150 mM-Na⁺

After desmin assembly in 150 mM-NaCl/10 mM-imidazole/HCl, pH 7.0, at 2 °C (■), at 22 °C (▲) or at 37 °C (●) reached maximum absorbance, samples were centrifuged at 12000 *g* (*r*_{av}, 59.1 cm) for 30 min, and the concentration of desmin remaining in the supernatant was determined as described in the Materials and methods section. Values are averages for four separate experiments.

desmin studies (Huiatt, 1979; Stromer *et al.*, 1987), in keratin assembly (Fukuyama *et al.*, 1978), in vimentin assembly (Hartzer, 1984) and in GFAP assembly (Yang & Babitch, 1988). Values for critical concentrations that were determined by the turbidity method with the three cations at 2 and 22 °C are compared with those obtained at 37 °C in Table 1.

Centrifugation

Critical concentration can also be defined as the minimum concentration required for initiating the IF assembly process. All protein in excess of the critical concentration will be assembled into filaments, but the amount of protein not assembled will remain virtually constant at the level of the critical concentration after assembly equilibrium is reached (Timasheff, 1981). The centrifugation method, therefore, is able to reveal critical concentration by separating filaments from a constant amount of desmin in the supernatant. The amount of desmin remaining in the supernatant will be the critical concentration for a specific set of conditions. Fig. 2 shows an average of four replications of a

centrifugation assay done in the presence of 150 mM-Na⁺ at 2, 22 and 37 °C. At each temperature, a nearly constant amount of desmin remains in the supernatant irrespective of the initial desmin concentration. The critical concentration of desmin required for assembly in the presence of 150 mM-Na⁺ is 51.8 μg/ml at 2 °C, 20.4 μg/ml at 22 °C and 8.5 μg/ml at 37 °C (Fig. 2). Critical concentrations obtained by the centrifugation assay in the presence of 2 mM-Ca²⁺ and 2 mM-Mg²⁺ at these temperatures are compared with those obtained with 150 mM-Na⁺ in Table 1.

Critical concentration

Critical concentrations obtained from both turbidity and centrifugation assays (Table 1) indicate that critical concentration decreases for each cation as temperature increases, and that this trend is very consistent with both methods. Because both methods show similar trends, we have averaged the values from the two methods to facilitate comparisons among the temperature-cation combinations (Table 1). Critical concentration is lowest in the presence of 2 mM-Ca²⁺ at each temperature. The critical concentration is highest in the presence of 150 mM-Na⁺ at 2 °C. When averaged over the three temperatures, the trend shows that critical concentrations are lower in the presence of the 2 mM bivalent cations than with 150 mM-Na⁺ (Table 1).

Negative staining

The distribution of diameters of particles remaining in the supernatant is shown in Table 2. As the temperature increases to 37 °C, the percentage of protofilaments (2.5 nm diameter) and protofibrils (5 nm diameter) decreases when compared with values obtained at 2 and 22 °C. Measurements of average diameters and lengths of particles that remained in the supernatant (Fig. 3a) after centrifugation are shown in Table 2. When averaged over all cations and temperatures, the diameter is about 5–6 nm, whereas the length is generally below 300 nm. Fully formed desmin filaments, however, have a diameter of about 10–11 nm and are several micrometres long. The particles remaining in the supernatant, therefore, are protofilaments, protofibrils or partly formed filaments in early stages of the assembly process (Fig. 3a). In addition, as temperature increases, average diameters and lengths of particles increase (Table 2). This suggests that higher temperatures favour a more extensive assembly process. On the other hand, the overall average diameter of filaments formed at 2, 22 or 37 °C and collected in the pellet (Fig.

Table 2. Diameter distribution, average diameter and average length of particles remaining in supernatant

Particle* diameter (nm)	Percentage in each range								
	2 mM-Ca ²⁺			2 mM-Mg ²⁺			150 mM-Na ⁺		
	2 °C	22 °C	37 °C	2 °C	22 °C	37 °C	2 °C	22 °C	37 °C
2.50 ± 1.25	35†	28	4	30	21	9	27	15	5
5.00 ± 1.25	60	56	30	65	64	39	42	40	35
7.50 ± 1.25	5	16	38	5	15	35	25	37	41
10.00 ± 1.25	—	—	28	—	—	17	6	8	19
Average diameter (nm)‡ ...	4.27 ± 1.41	4.70 ± 1.63	7.37 ± 2.30	4.38 ± 1.36	4.85 ± 1.50	6.57 ± 2.23	5.25 ± 2.15	5.97 ± 2.07	6.88 ± 2.08
Average length (nm)§ ...	105 ± 57	110 ± 67	158 ± 69	129 ± 68	122 ± 64	209 ± 95	223 ± 94	273 ± 120	326 ± 120

* Particles are grouped by diameter into four ranges.

† Each percentage is calculated on the basis of 150 measurements.

‡ Each mean value with its S.E.M. is calculated from 150 measurements.

§ Each mean value with its S.E.M. is calculated from 120 measurements.

Fig. 3. Negatively stained samples from the centrifugation assay

(a) Supernatants from each temperature-cation combination typically contain protofilaments (arrow), short aggregates (double arrow) and partly formed desmin filaments with average lengths < 300 nm and with average diameters of 5–6 nm. These particles were in the supernatant of a sample assembled with 2 mM-MgCl₂/10 mM-imidazole/HCl, pH 7.0, at 2 °C and centrifuged at 40000 *g* (r_{av} , 59.1 cm) for 30 min. (b) Filaments in the pellet from a sample assembled with 150 mM-NaCl/10 mM-imidazole/HCl, pH 7.0, at 37 °C and centrifuged at 12000 *g* (r_{av} , 59.1 cm) for 30 min show some lateral aggregation. Individual filaments, however, have average diameters of 10 nm, a value typical of all pelleted filaments in these experiments. The bar represents 100 nm.

Table 3. Average diameter (nm) of desmin filaments in pellet

Each mean value with its S.E.M. is calculated from 150 measurements.

Temperature (°C)	Average diameter (nm)		
	2 mM-Ca ²⁺	2 mM-Mg ²⁺	150 mM-Na ⁺
2	10.00 ± 1.60	9.75 ± 1.62	9.63 ± 1.40
22	10.02 ± 1.44	9.78 ± 1.29	9.77 ± 1.34
37	11.13 ± 2.07	10.33 ± 1.44	10.30 ± 1.60

3b) is very close to 10 nm (Table 3). The filaments in Fig. 3(b) were assembled in the presence of 150 mM-Na⁺ at 37 °C and show some lateral aggregation, which is common when high salt and higher temperatures are used. Filament diameters were measured only where the edges of single filaments could be unambiguously recognized. Although length of individual filaments in the pellet could usually not be determined because of tangling of the filaments, many could be followed accurately for more than 1 μ m (results not shown).

Thermodynamic data

Critical concentration can also be defined as the reciprocal of the equilibrium constant (K_{eq}) of the nucleation-elongation assembly reaction (Timasheff, 1981). Therefore the free energy

(ΔG), enthalpy (ΔH) and entropy (ΔS) for assembly can be estimated from the van't Hoff equation (Chang, 1977):

$$\ln C = -\ln K_{eq} = (-\Delta H/R)(1/T) + \Delta S/R$$

where C is the critical molar concentration, R is the universal gas constant and T the temperature (in K). ΔH is estimated from the slope of the van't Hoff plot of the natural logarithm of the critical molar concentration versus $1/T$, and ΔS is estimated from the y -intercept. Then ΔG is directly calculated from the relationship:

$$\Delta G = \Delta H - T\Delta S$$

The results (Table 4), which were calculated from the average values in Table 1, indicate that desmin assembly, in the presence of each cation, is endothermic ($\Delta H > 0$) and is driven by entropy. This implies that hydrophobic interactions are dominant during desmin assembly in the presence of these cations (Cantor & Schimmel, 1980).

DISCUSSION

Turbidity

The validity of using turbidity to measure the weight concentration of particles in suspension is based on both theoretical calculations (Berne, 1974) and experimental evidence (Gaskin *et al.*, 1974). Turbidity is a function of the total concentration of scattering particles and is independent of the length distribution of the particles (Berne, 1974; Gaskin *et al.*, 1974). There are,

Table 4. Thermodynamic data of desmin assembly in the presence of cations

van't Hoff plots were calculated from the values in Table 2. ΔH with its S.E.M. is estimated from the slope of the plot, and ΔS with its S.E.M. is estimated from the h -intercept. ΔG is calculated from the relationship:

$$\Delta G = \Delta H - T\Delta S$$

Cation present ... van't Hoff plot ...	2 mM-Ca ²⁺ $\ln C = 3183(1/T) - 26$ $r = 0.956$	2 mM-Mg ²⁺ $\ln C = 1978(1/T) - 21$ $r = 0.955$	150 mM-Na ⁺ $\ln C = 4002(1/T) - 28$ $r = 0.990$
Parameter			
ΔH (kJ/mol)	+26.5 ± 8.0	+16.4 ± 5.0	+33.6 ± 5.0
ΔS (J · mol ⁻¹ · K ⁻¹)	+218 ± 25	+176 ± 17	+235 ± 17
ΔG_{275} (kJ/mol)	-33.6	-32.3	-31.1
ΔG_{295} (kJ/mol)	-37.8	-35.7	-35.7
ΔG_{310} (kJ/mol)	-41.2	-38.2	-39.9

however, two requirements that need to be met before using the turbidity assay: (1) the length (L) of rod-like particles should be large in comparison with the wavelength (λ) of light used for measurement, and (2) turbidity should be proportional to the inverse third power of the wavelength (λ^{-3}). Timasheff (1981) has stated that turbidity assays are valid if the ratio of particle length (L) to the wavelength used is greater than 3.5. Our results show that most filaments in our samples are longer than 1 μ m, and that would provide a ratio of L/λ_{300} of more than 3.5. Reassembled desmin filaments, therefore, meet the rod-like requirement. This conclusion is consistent with our previous study (Huiatt, 1979) and with the work of Steinert *et al.* (1976) on keratin filaments. Referring to the second point, Camerini-Otero & Day (1978) suggested that presence of aberrant structures or non-ideality of the solution might be responsible for a deviation of the wavelength exponent from the theoretical value of -3 . Zackroff & Goldman (1979) also suggested that the wavelength exponent of reassembled vimentin filaments might be influenced by the pH and ionic strength of the filament-forming buffer. Our results (not shown), however, indicated that the dependence of turbidity on wavelength gave wavelength exponents close to -3 for each temperature-cation combination tested. In addition, results from this study and others (Huiatt, 1979; Steinert *et al.*, 1976) demonstrated that, for each temperature-cation combination, turbidity in the plateau region was linearly related to total protein concentration and was independent of the length distribution of particles. Although the turbidity method has limitations (Timasheff & Grisham, 1980), results from our laboratory and from others indicate that it is an appropriate assay for measuring protein assembly.

Centrifugation

Centrifugation was used in the present study to pellet desmin filaments and to leave a constant amount of desmin in the supernatant. For this approach to be valid, the following criteria should be met: (1) the rate of disassembly must be low relative to the duration of centrifugation so that the equilibrium distribution is not disturbed during the course of the experiment; and (2) the assembly equilibrium should not be affected significantly by pressure (Timasheff, 1981). Timasheff (1981) pointed out that the second criterion might be very difficult to meet. In the case of actin assembly, Ikkai & Ooi (1966) demonstrated that high pressure generated near the bottom of the tubes during centrifugation shifted the assembly equilibrium of actin to the monomer state and finally induced irreversible denaturation of the actin monomer. It was suggested that assembly was associated with an increase in the volume of the actin solution; that is, the difference in partial specific volume between polymer and

monomer ($\Delta\bar{v}$) was changed (Ikkai *et al.*, 1966). Because many assembly processes are accompanied by a significant value of $\Delta\bar{v}$ (Harrington & Kegeles, 1973), desmin assembly may also be affected by pressure. Unfortunately, there are no published data on this aspect of desmin assembly.

The criteria used to select the centrifugal force used in our experiments were based on a report by Timasheff (1981) and on previous studies from our laboratory. Timasheff (1981) proposed two criteria for determining whether the appropriate centrifugal force has been used. First, the concentration of protein in the supernatant should be comparable with the critical concentration determined by another method. Second, the protein remaining in the supernatant must be unable to assemble at atmospheric pressure. We obtained similar values for critical concentrations by two independent methods, namely turbidity and centrifugation (Table 1). Negative staining of aliquots of supernatants immediately after centrifugation and 40 or 60 min after centrifugation showed that particle length did not increase as time after centrifugation increased (results not shown). This indicated that the amount of desmin remaining in the supernatant did not exceed the critical concentration.

Our laboratory had previously developed a low speed (12000 g , r_{av} . 59.1 cm) centrifugation method to determine the effect of ionic strength on desmin and vimentin assembly. Huiatt *et al.* (1980) demonstrated that over 95% of desmin could be pelleted as filaments during a 30 min spin if assembly was done in the presence of 150 mM-Na⁺ at 2 °C. Negative staining showed that pellets contained long filaments with average diameters of 10–11 nm, but that supernatants contained protofilaments, protofibrils and short (< 300 nm) particles (Hartzer, 1984). On the basis of these observations, 12000 g (r_{av} . 59.1 cm) was selected to reveal critical concentration required for desmin assembly in the presence of 150 mM-Na⁺ at 2 °C. This centrifugal force was also used to reveal critical concentrations in the presence of 150 mM-Na⁺ at 22 and 37 °C and of 2 mM-Ca²⁺ or Mg²⁺ at 37 °C, because the turbidities observed for assembling a given amount of protein were higher with those temperature-cation combinations than in the presence of 150 mM-Na⁺ at 2 °C. The 12000 g force was initially tried with the 2 mM-Ca²⁺ and with the 2 mM-Mg²⁺ samples at 2 and at 22 °C, but the desmin concentration in the supernatant did not remain constant as the starting desmin concentration was increased (results not shown). A greater centrifugal force (40000 g , r_{av} . 59.1 cm), therefore, was needed to reveal critical concentration in the presence of 2 mM-Ca²⁺ or -Mg²⁺ at 2 or 22 °C. The criterion of leaving a constant concentration of desmin in the supernatant independent of the initial desmin concentration was met by using this higher centrifugal force.

Factors affecting particle morphology

Our laboratory previously investigated the effects of pH and concentration of Mg^{2+} or Na^+ on yield and morphology of desmin intermediate filaments (Huiatt, 1979). The optimum pH for assembly of desmin filaments was 7, the same as that utilized in these experiments. The studies by Huiatt (1979) also demonstrated that ionic strength had no effect on dimensions of the protofilaments. A comparison of desmin filaments formed in 10 mM-imidazole/HCl, pH 7, with those formed in the same buffer plus 150 mM-NaCl showed that the major effect of increasing ionic strength at pH 7 was to increase filament length. The purpose of the experiments described in the present paper was to determine the critical concentration required for desmin assembly at pH 7 in the presence of two bivalent cations or a frequently utilized univalent cation at three temperatures. On the basis of our previous studies and on the results shown in Table 2, an increase in ionic strength is associated with an increase in filament length. Data shown in Table 3 indicate, however, that there is little variation in filament diameters for all experimental conditions tested and that differences in ionic strength had little effect on diameter of filaments.

Factors affecting critical concentration

Our data show that both temperature and cations modify the critical concentration of desmin. Between 2 and 37 °C, critical concentration decreases as temperature increases. This implies that higher temperature favours more extensive assembly because of higher K_{eq} . This is consistent with our previous results showing that desmin assembly increased as temperature increased from 2 to 37 °C (Stromer *et al.*, 1987). Secondly, our results show that, when averaged over the three temperatures, critical concentration for desmin assembly in the presence of 2 mM bivalent cations is lower than in the presence of 150 mM- Na^+ , a univalent cation frequently used at this concentration for assembly (Huiatt *et al.*, 1980; Inagaki *et al.*, 1988). This also agrees with our previous studies (Stromer *et al.*, 1987) and with studies on other IF proteins (Fukuyama *et al.*, 1978; Yang & Babitch, 1988). Yang *et al.* (1988) have demonstrated that there are three classes of bivalent-cation-binding sites, one of which is Ca^{2+} -specific and is in the rod domain of GFAP protein. Because the rod domains of IF proteins are the most highly conserved regions of the molecules, it is possible that Ca^{2+} -binding sites may also exist in desmin and that binding of Ca^{2+} may cause a molecular change that favours filament assembly. Our data suggest that desmin assembly is a complex, multistep, process. One possible interpretation of the data in Table 2 is that Na^+ favours filament elongation and that Ca^{2+} and Mg^{2+} at 2 and 22 °C favour nucleation.

Recent studies by Inagaki *et al.* (1988) have shown that phosphorylation *in vitro* of desmin tetramers inhibits the assembly process and that phosphorylation *in vitro* of desmin filaments causes disassembly. Geisler & Weber (1988) determined that the three phosphorylation sites on desmin are in the N-terminal head domain, the region that has an important functional role in filament assembly (Traub & Vorgias, 1983, 1984; Kaufmann *et al.*, 1985; van den Heuvel *et al.*, 1987). The desmin used in the studies reported here was prepared by the method of Huiatt *et al.* (1980) and contained 20% α - (more acidic) and 80% β -desmin variants. Although phosphorylation affects assembly, all of our preparations contained a consistent ratio of α and β variants, thus making comparisons among the treatments valid. If phosphorylation, by itself, controls desmin assembly, when the initial desmin concentration and thus the amount of the α variant is increased in an assembly assay, there should be some increase in the amount of unassembled desmin remaining in the supernatant.

The results shown in Fig. 2 indicate that this did not occur. This may indicate that the predominant β variant can serve as a template for rod-to-rod associations that incorporate both α - and β -desmin into the assembling particles.

Forces involved in assembly

Theoretical calculations of possible ionic interactions between the coiled-coil rods of IF proteins (Crewther *et al.*, 1983; Fraser *et al.*, 1985; Conway & Parry, 1988) formed the basis for the concept that ionic interactions are the key force responsible for alignment and registry of dimers in the IF. Our thermodynamic data indicate that desmin assembly in the presence of cations is endothermic and is driven by entropy. This implies that hydrophobic rather than ionic interactions are dominant during assembly, especially in the presence of high salt (because of charge masking by cations). This conclusion is supported by the fact that higher ionic strength favours more extensive assembly of IF proteins (Huiatt, 1979; Huiatt *et al.*, 1980; Renner *et al.*, 1981; Steinert *et al.*, 1981; Nelson & Traub, 1982; Hartzer, 1984; Ip *et al.*, 1985a,b; Stromer *et al.*, 1987). Hatzfeld *et al.* (1987) also demonstrated that formation of the heterotypic cytokeratin complex did not depend solely on ionic interactions. Our data do not address the question of involvement of ionic interactions in alignment and registry of subunits, but suggest that hydrophobic interactions are the major force for desmin IF assembly in the presence of cations.

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REFERENCES

- Aebi, U., Fowler, W. E., Rew, P. & Sun, T.-T. (1983) *J. Cell Biol.* **97**, 1131–1143
- Berne, B. J. (1974) *J. Mol. Biol.* **89**, 755–758
- Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254
- Camerini-Otero, R. D. & Day, L. A. (1978) *Biopolymers* **17**, 2241–2249
- Cantor, C. R. & Schimmel, P. R. (1980) *Biophysical Chemistry: Part I: The Conformation of Biological Macromolecules*, pp. 253–309, W. H. Freeman and Co., San Francisco
- Chang, R. (1977) *Physical Chemistry with Applications to Biological Systems*, p. 243, Macmillan Publishing Co., New York
- Conway, J. F. & Parry, D. A. D. (1988) *Int. J. Biol. Macromol.* **10**, 79–88
- Crewther, W. G., Dowling, L. M., Steinert, P. M. & Parry, D. A. D. (1983) *Int. J. Biol. Macromol.* **5**, 267–274
- Fraser, R. D. B., Macrae, T. P., Suzuki, E. & Parry, D. A. D. (1985) *Int. J. Biol. Macromol.* **7**, 258–274
- Fukuyama, K., Murozuka, T., Caldwell, R. & Epstein, W. L. (1978) *J. Cell Sci.* **33**, 255–263
- Gaskin, F., Cantor, C. R. & Shelanski, M. L. (1974) *J. Mol. Biol.* **84**, 739–758
- Geisler, N. & Weber, K. (1982) *EMBO J.* **1**, 1649–1656
- Geisler, N. & Weber, K. (1988) *EMBO J.* **7**, 15–20
- Harrington, W. F. & Kegeles, G. (1973) *Methods Enzymol.* **27**, 306–345
- Hatzfeld, M., Maier, G. & Franke, W. W. (1987) *J. Mol. Biol.* **197**, 237–255
- Hartzer, M. K. (1984) Ph.D. Dissertation, Iowa State University, Ames
- Huiatt, T. W. (1979) Ph.D. Dissertation, Iowa State University, Ames
- Huiatt, T. W., Robson, R. M., Arakawa, N. & Stromer, M. H. (1980) *J. Biol. Chem.* **255**, 6981–6989
- Ikkai, T. & Ooi, T. (1966) *Biochemistry* **5**, 1551–1560

- Ikkai, T., Ooi, T. & Noguchi, H. (1966) *Science* **152**, 1756–1757
- Inagaki, M., Gonda, Y., Matsuyama, M., Nishizawa, K., Nishi, Y. & Sato, C. (1988) *J. Biol. Chem.* **263**, 5970–5978
- Ip, W., Hartzler, M. K., Pang, Y.-Y. S. & Robson, R. M. (1985a) *J. Mol. Biol.* **183**, 365–375
- Ip, W., Heuser, J. E., Pang, Y.-Y. S., Hartzler, M. K. & Robson, R. M. (1985b) *Ann N.Y. Acad. Sci.* **455**, 185–199
- Kaufmann, E., Weber, K. & Geisler, N. (1985) *J. Mol. Biol.* **185**, 733–742
- Nelson, W. T. & Traub, P. (1982) *J. Cell Sci.* **53**, 77–95
- O'Shea, J. M., Robson, R. M., Hartzler, M. K., Huiatt, T. W., Rathbun, W. E. & Stromer, M. H. (1981) *Biochem. J.* **195**, 345–356
- Pang, Y.-Y. S. & Robson, R. M. (1984) *J. Cell Biol.* **99**, 320a
- Pang, Y.-Y. S., Robson, R. M., Hartzler, M. K. & Stromer, M. H. (1983) *J. Cell Biol.* **97**, 226a
- Renner, W., Franke, W. W. & Schmid, E. (1981) *J. Mol. Biol.* **149**, 285–306
- Robson, R. M. (1989) *Curr. Opin. Cell Biol.* **1**, 36–43
- Steinert, P. M. & Roop, D. R. (1988) *Annu. Rev. Biochem.* **57**, 593–625
- Steinert, P. M., Idler, W. W. & Zimmerman, S. B. (1976) *J. Mol. Biol.* **108**, 547–567
- Steinert, P. M., Idler, W. W., Cabral, F., Gottesman, M. M. & Goldman, R. D. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3692–3696
- Stromer, M. H. (1990) in *Cellular and Molecular Biology of Intermediate Filaments* (Goldman, R. D. & Steinert, P. M., eds.), pp. 19–36, Plenum Publishing Corp., New York
- Stromer, M. H., Huiatt, T. W., Richardson, F. L. & Robson, R. M. (1981) *Eur. J. Cell Biol.* **25**, 136–143
- Stromer, M. H., Ritter, M. A., Pang, Y.-Y. S. & Robson, R. M. (1987) *Biochem. J.* **246**, 75–81
- Timasheff, S. N. (1981) in *Protein-Protein Interactions* (Frieden, C. & Nichol, L. W., eds.), pp. 315–336, John Wiley and Sons, New York
- Timasheff, S. N. & Grisham, L. M. (1980) *Annu. Rev. Biochem.* **49**, 565–591
- Traub, P. & Vorgias, C. E. (1983) *J. Cell Sci.* **63**, 43–67
- Traub, P. & Vorgias, C. E. (1984) *J. Cell Sci.* **65**, 1–20
- van den Heuvel, R. M. M., van Eys, G. J. J. M., Ramaekers, F. C. S., Quax, W. J., Vree Egberts, W. T. M., Schaart, G., Cuypers, H. T. M. & Bloemendal, H. (1987) *J. Cell Sci.* **88**, 475–482
- Vorgias, C. E. & Traub, P. (1983) *Prep. Biochem.* **13**, 227–243
- Yang, Z. W. & Babitch, J. A. (1988) *Biochemistry* **27**, 7038–7045
- Yang, Z. W., Chung, F. K. & Babitch, J. A. (1988) *Biochemistry* **27**, 7045–7050
- Zackroff, R. V. & Goldman, R. D. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 6226–6230

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