Effect of cations and temperature on kinetics of desmin assembly

Marvin H. STROMER,* Margaret A. RITTER, Y.-Y. Susana PANG and Richard M. ROBSON

Muscle Biology Group, Departments of Animal Science, Biochemistry and Biophysics and Food Technology, Iowa State University, Ames, IA 50011, U.S.A.

Smooth-muscle desmin, which was isolated from avian gizzard, was purified and used to form reconstituted intermediate filaments. Filament assembly was done in the presence of physiological cations, Ca^{2+} , Mg^{2+} , Na^+ , and Na^+ plus Mg^{2+} , and with non-physiological cations Cu^{2+} and Ni^{2+} . Assembly was done at 2°, 22° and 37 °C, and was monitored by absorbance and by electron microscopy. Absorbance increased most rapidly during the first 2–5 min and then increased at a slower rate with the physiological cations, but decreased after that time with the non-physiological cations. For each physiological cation, absorbance increased with increasing temperature. This was particularly evident with Ca^{2+} , which produced the lowest absorbance at 2 °C and the highest at 37 °C. When ionic strength was comparable, filament-forming buffers that contained bivalent cations were associated with higher absorbance values. Filament diameters were significantly smaller 60 min after assembly initiation than after 5 min. Average filament diameters, when formed in the presence of Cu^{2+} or Ni^{2+} , were 10% greater than in the presence of the physiological cations and did not show a consistent tendency to decrease as time increased. These results demonstrate the importance, not only of the pH and ionic composition of the filament-forming buffer, but also of the temperature and duration of dialysis for reconstitution of desmin filaments.

INTRODUCTION

Many eukaryotic cells contain cytoplasmic filaments that have average diameters of 10 nm and are usually referred to as 'intermediate' or '10 nm' filaments. On the basis of their immunological properties and their mobility in one- or two-dimensional gel electrophoresis, these filaments have been divided into the following five classes: cytokeratin or keratin (epithelial-type cells), vimentin (several cell types but mainly in mesenchymally derived cells), neurofilaments (neurons), glial (cells of glial origin) and desmin (smooth- and striated-muscle cells) (Lazarides, 1982; Osborn & Weber, 1982; Osborn *et al.*, 1982). The protein constituents of those filaments have been studied in many laboratories, and amino acid sequences have been determined (Geisler & Weber, 1981, 1982, 1983; Geisler *et al.*, 1982; Steinert *et al.*, 1983) for all classes of 10 nm filaments. As a group, 10 nm filaments are relatively insoluble and require denaturing conditions, e.g. low pH (O'Shea *et al.*, 1981) or urea (Huiatt *et al.*, 1980), to solubilize the filaments. Once it has been isolated and purified, smooth-muscle desmin will, however, remain soluble in the absence of the denaturant so long as the ionic strength is low (< 0.01) and the pH is ≥ 8.5 .

The structure and substructure of 10 nm filaments have been studied in various ways. Stromer *et al.* (1981) used urea or low ionic strength to disassemble 10 nm filaments formed *in vitro* from purified smooth-muscle desmin. Negative staining of the disassembly products revealed 2–2.5 nm-diameter protofilaments and 4–4.5 nm-diameter subfilaments that appeared to be composed of two protofilaments. Aebi *et al.* (1983) used phosphate ions, lowered pH and trypsin treatment to show that reconstituted epidermal keratin filaments also consisted of 2 nm protofilaments and 4.5 nm 'protofibrils' that frequently displayed a 5.4 nm axial repeat. On the basis of mapping of peptides produced by CNBr

treatment and of trypsin hydrolysis of 10 nm-filament proteins from epidermal keratinocytes and from BHK-21 cells, Steinert *et al.* (1980) proposed a three-stranded coiled-coil model for the protofilament. More recently, a dimeric double-stranded coiled-coil protofilament model has been proposed on the basis of amino acid sequence data (Geisler & Weber, 1982) and of cross-linking experiments with desmin (Pang *et al.*, 1983; Pang & Robson, 1984). Enzymic removal of non-helical segments of the coiled coil indicated that these segments are involved in the assembly *in vitro* of vimentin (Traub & Vorgias, 1983) and of cytokeratin filaments (Sauk *et al.*, 1984). Removal of a 27-residue segment from the C-terminal end of chicken gizzard desmin, however, did not prevent the remaining fragment from assembling into filaments (Kaufmann *et al.*, 1985). Transverse periodicities in 10 nm keratin filaments have been ascribed to helical domains (Henderson *et al.*, 1982) and, in 4.5 nm keratin protofibrils, either to helical organization or to protofilament packing (Aebi *et al.*, 1983).

Reconstitution of 10 nm filaments *in vitro* from purified proteins usually involves increasing the ionic strength, or lowering the pH to near neutrality, or both. Assembly of desmin, for example, is typically initiated *in vitro* by increasing the ionic strength to 0.1 by adding bivalent or univalent cations and/or by decreasing the pH to 7.0. There are, however, no published data on the influence of specific cations, of temperature and of time in dialysis on the kinetics of 10 nm-filament assembly. Because of evidence from previous studies in our laboratory (Huiatt, 1979) that MgCl_2 was a more effective promoter of desmin assembly at 2 °C than was NaCl , we specifically compared the effects of cations, of temperature and of time in dialysis on the kinetics of 10 nm-filament assembly. The second objective of the present study was to determine if cations, temperature or time of assembly would alter filament diameter or length. This information would facilitate the choice of either a

* To whom reprint requests should be addressed.

known set of conditions for filament reconstitution or of selectively deviating from optimal conditions for studying assembly intermediates. We have assembled filaments from highly purified smooth-muscle desmin at three temperatures and in the presence of four physiologically relevant cations, or combinations thereof, and of two non-physiologically relevant cations. The non-physiological cations, Cu^{2+} and Ni^{2+} , were included because of their demonstrated ability to disrupt microtubules (Roth & Shigenaka, 1970) and because of the reported relationships between microtubules and 10 nm filaments (Goldman & Follet, 1969; Rice *et al.*, 1980; Blose, 1981; Runge *et al.*, 1981; Schliwa & Van Blerkom, 1981; Maro *et al.*, 1983).

EXPERIMENTAL

Preparation of desmin

Desmin was extracted and purified from fresh turkey gizzards by the method of Huiatt *et al.* (1980). Each of the four desmin preparations used was characterized by SDS/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969). Urea was removed from the purified desmin by dialysis against several changes of 10 mM-Tris/acetate, pH 8.5. Before samples were used for an experiment, they were dialysed against 1 mM- NaHCO_3 , pH 8.0, for 4 h at 2 °C and were clarified by centrifugation at 40000 rev./min for 40 min in a Beckman Ti 70.1 rotor. Protein was determined in the supernatants by the biuret method (Gornall *et al.*, 1949) as modified (Robson *et al.*, 1968).

Spectrophotometry of desmin

Measurements were done with a Zeiss PMQII spectrophotometer at 300 nm with a 5.5–6.0 nm spectral slit width. Temperature was maintained during the experiment by pumping coolant from a Lauda circulator through the sample chamber. Solutions were degassed before use to limit bubble formation in the cuvette, and were equilibrated to the temperature of the experiment (2, 22 or 37 °C) before use. Control solutions of monomeric desmin (0.2 mg/ml) in 1 mM- NaHCO_3 , pH 8.0, were used to zero the spectrophotometer. Assembly of desmin was initiated in the cuvette by adding 0.5 ml of filament-forming buffer [60 mM-imidazole/HCl, pH 7.04, plus appropriate concentrations of the respective cation(s)] to 1 ml of desmin (diluted to 0.3 mg/ml with 1 mM- NaHCO_3 , pH 8.0). The solution was mixed by drawing the mixture twice into a Pasteur pipette. Final assembly conditions were: 0.2 mg of desmin/ml in 20 mM-imidazole/HCl, pH 7.04, which also contained 5 mM- CaCl_2 , or 5 mM- MgCl_2 , or 150 mM- NaCl , or 5 mM- MgCl_2 plus 150 mM- NaCl , or 5 mM- NiCl_2 , or 2 mM- CuCl_2 .

Negative staining

Samples of all preparations were negatively stained 5 min and 60 min after assembly was started. Immediately after mixing the sample in the cuvette (see under 'Spectrophotometry of desmin'), a two-drop sample was removed and placed in a test tube incubated at the temperature of the experiment. A drop of the sample was added to a glow-discharged carbon-coated grid 5 min after assembly was initiated. After 60 s, the grid was washed with five drops of glass-distilled water and was stained with 2% uranyl acetate. The 60 min sample was

Fig. 1. Absorbance of desmin in the presence of Ca^{2+} at 2, 22 and 37 °C

Desmin was assembled in 5 mM- MgCl_2 /20 mM-imidazole/HCl, pH 7.04. Values from four preparations were averaged. Bars indicate standard errors.

removed from the cuvette after mixing with a Pasteur pipette and was stained in the same way.

Electron microscopy

Negatively stained samples were examined in a JEOL JEM-100CXII electron microscope operated at 80 kV. Images were recorded on Kodak SO-163 electron image film at nominal magnifications of $\times 27000$ or 50000. Actual magnifications were determined by photographing a carbon-grating replica (E. F. Fullam Inc., Latham, NY, U.S.A.).

Filament measurement

Micrographs from the four repetitions of each treatment were randomized, and ten micrographs were drawn at random for each treatment. Filaments to be measured were identified by randomly placing a clear acetate sheet with lines 1 cm apart over the micrograph. Filament diameters were measured as near as possible to the intersection of the grid line and the filament. An average of ten diameters were measured per micrograph, and a minimum of 100 were done per treatment with each of the physiologically relevant cations. Because of clumping and lateral aggregation in the Ni^{2+} - and Cu^{2+} -treated samples, only 25–50 diameters could be measured, except in two of the Ni^{2+} -treated samples, in which only ten could be measured. Length measurements were possible only on desmin preparations exposed to the physiologically relevant cations. Fewer measurements were possible as time and temperature increased for a particular cation, because of concomitant increases in length and tangling, which made it increasingly difficult to determine unequivocally the ends of the filament. A Bioquant Image Analysis System (R&M Biometrics, Nashville, TN, U.S.A.) was used for the measurements.

RESULTS

Effect of physiological cations and temperature on desmin assembly

(a) **Absorbance of desmin.** The absorbance of desmin assembled in the presence of 5 mM- CaCl_2 at 2, 22 or 37 °C is shown in Fig. 1. In general, the results obtained with the other physiological cations showed similarly shaped curves and temperature responses. To determine which of the physiological cations is the most effective

Table 1. Initial rate of desmin assembly and absorbance at 60 min

Initial rate is defined as absorbance change per min during the first 5 min of assembly; initial rates within a row having different superscripts are significantly different. Absorbances at 60 min within a row having different superscripts are significantly different.

Cation	Temp. (°C) ...	10 ³ × Initial rate			10 ³ × Absorbance at 60 min		
		2	22	37	2	22	37
Physiological							
CaCl ₂		4 ^a	9 ^b	31 ^c	25 ^d	69 ^e	225 ^f
MgCl ₂		7	8	19 ^a	36 ^b	61 ^c	132 ^d
NaCl		6	7	9 ^a	33 ^b	43 ^c	62 ^d
Na ⁺ + Mg ²⁺		6	8	12	35	54	82
Non-physiological							
CuCl ₂		48	57	90 ^a	191	203	360 ^b
NiCl ₂		58	60	64	242	242	269

potentiator of desmin assembly, absorbances for a cation were averaged over all three temperatures (results not shown). A filament-forming buffer containing 5 mM-CaCl₂ produces a significantly greater response ($P < 0.05$) than the other three cations. Absorbance is also significantly higher ($P < 0.05$) when filaments are assembled in the presence of 5 mM-MgCl₂ than with 150 mM-NaCl. The average absorbance of filaments assembled in Na⁺ plus Mg²⁺ is intermediate between the absorbances for Mg²⁺ and for Na⁺ alone, and does not differ significantly from either. The temperature effect was similarly determined by averaging absorbances over the four physiological cations (results not shown). This approach shows that, as temperature increases, significant increases ($P < 0.05$) in absorbance are observed. The interaction of a cation and temperature can most readily be quantitatively compared with all other cation/temperature combinations by determining the slope and intercept for each combination. The method of Tukey *et al.* (1985) was used to obtain the slopes and intercepts (results not shown). For all physiological cations, the intercept increases as the temperature increases and, except for Na⁺, the slope also increases. In addition, the intercepts and slopes of the CaCl₂/37 °C and the MgCl₂/37 °C lines are much higher than for any other physiological cation/temperature combination. This shows that, at 37 °C, these two bivalent cations are more effective promoters of desmin assembly than is any other combination that we investigated. Table 1 shows both the initial rate of assembly and the absorbance observed after 60 min of assembly. These values indicate that CaCl₂ is the least effective physiological potentiator of assembly at 2 °C, but is the most effective at 37 °C.

(b) Electron microscopy of desmin. Desmin filaments negatively stained after 5 min assembly at 2 °C in the presence of 5 mM-CaCl₂ are shown in Fig. 2(a). Mixtures of short filaments and aggregates of protofilaments characterize these samples. Longer filaments and fewer protofilamentous aggregates are evident in Fig. 2(b), a sample assembled in the presence of 5 mM-CaCl₂ for 5 min at 37 °C. After 60 min at 2 °C in the presence of 5 mM-CaCl₂, long filaments predominate (Fig. 2c), but some short filamentous particles are still present. When compared with Fig. 2(a), it is clear that lengthening of

filaments and maturation of the filamentous particles have occurred.

At 5 min after initiation of assembly with 150 mM-NaCl at 2 °C, the sample consists mostly of short filaments and filamentous particles, but also contains a few longer filaments (Fig. 2d). Long filaments, which sometimes have a 20–22 nm transverse periodicity, are the predominant structure seen if desmin is challenged for 5 min with 150 mM-NaCl at 37 °C (Fig. 2e). The long filaments formed in the presence of 150 mM-NaCl for 60 min at 2 °C (Fig. 2d) and the virtual absence of filamentous particles again indicate that a lengthening process has been the primary structural event.

Table 2 shows a comparison of filament diameters for each time/temperature/cation combination. Although all three variables affect diameters, temperature and time have the greatest affect. The three temperature means near the bottom of the Table show that significantly ($P < 0.05$) larger diameters are observed at 37 °C than at 2 or 22 °C. The exception to this occurred with NaCl, where no increase in diameter was noted with increased temperature. Except with MgCl₂ at 22 °C, diameters are always smaller at 60 min than at 5 min. The two time means at the bottom of Table 2 show that, when averaged over all cations and temperatures, mean filament diameters at 5 min (14.9 nm) are significantly larger ($P < 0.05$) than at 60 min (13.6 nm). The cation means in the right column indicate that CaCl₂ and MgCl₂ produce larger diameters than do either NaCl or NaCl plus MgCl₂.

Measurements of filament length are consistent with a mechanism of filament elongation as time and temperature increase. When averaged over all cations and temperatures, filaments are nearly twice as long (519 nm versus 265 nm) at 60 min as at 5 min (results not shown). If the data are averaged over the four cations and the two times, it is clear that filament length also increases with increasing temperature and that the increment of added length is greatest when assembly is done at 37 °C.

Effect of non-physiological cations and temperature on desmin assembly

(a) Absorbance of desmin. The absorbance of desmin assembled in the presence of 2 mM-CuCl₂ at 2, 22 or

Table 2. Diameters of reconstituted desmin filaments

Each value in the body of the Table is the average of 100 observations. Values in rows or columns of the body of the table differing by 1.8 or more are significantly different ($P < 0.05$; Student's t test). Different superscripts among either the four overall cation means (LSD = 0.7) or the three overall temperature means (least significant difference = 0.6) indicate a significant difference ($P < 0.05$, Student's t test). The two time means below the Table each are the average of 1200 measurements, are averaged over three temperatures and four cations and are significantly different (LSD = 0.2; $P < 0.05$; Student's t test).

Cation	Temp. (°C) . . . Time . . .	Diameter (nm)						Cation means
		2		22		37		
		5 min	60 min	5 min	60 min	5 min	60 min	
CaCl ₂		13.5	13.0	14.9	13.7	17.9	15.0	14.7 ^a
MgCl ₂		15.0	13.9	13.3	13.9	16.1	15.2	14.5 ^{a, b}
NaCl		15.8	13.0	14.5	12.9	14.2	13.2	13.9 ^b
Na ⁺ + Mg ²⁺		13.6	12.9	14.3	13.4	15.8	14.1	14.0 ^b
Temperature time means		14.5	13.3	14.2	13.2	16.0	14.4	14.3
Temperature means		13.9		13.8		15.2 ^a		
		Time		Mean				
		5 min		14.9				
		60 min		13.6				

37 °C is shown in Fig. 3. Absorbance at 37 °C is significantly higher than at 2° or 22 °C in the presence of CuCl₂ and, contrary to the pattern observed with physiological cations, decreases after 5 min. A similar pattern was observed when desmin was assembled in the presence of 5 mM-NiCl₂, except that no significant temperature effects were noted. Table 1 shows that both the initial rate and the absorbance at 60 min are greater for the non-physiological cations than for the physiological cations.

(b) Electron microscopy of desmin. Desmin filaments reconstituted in the presence of either CuCl₂ or NiCl₂ not only form very rapidly, but also clump very severely. For this reason, fewer diameters could be measured reliably, and length measurements were not possible. Samples negatively stained 5 min after assembly initiation contain only tangled clumps of long filaments and none of the aggregates characteristic of intermediate stages of assembly. The flocculent material at the bottom of the cuvette consisted entirely of clumped filaments. Diameters of filaments formed in the presence of CuCl₂ are slightly larger than, but very similar to, those formed in the presence of CaCl₂ or MgCl₂ (results not shown). The average diameter of filaments formed in the presence of

NiCl₂, however, is 1.8 nm larger than with CuCl₂. The effect of time on filament diameter did not show the consistent trend noted with the physiological cations.

DISCUSSION

This study utilized absorbance measurements and negative staining of the assembly products of smooth-muscle desmin to determine the effects of cations, temperature and time on kinetics of assembly and on filament diameter. The cation concentrations chosen were based on previous studies in our laboratory (Huiatt, 1979; Huiatt *et al.*, 1980), which showed that the greatest yield of desmin filaments could be obtained at pH 7.0 between 130 mM- and 160 mM-NaCl. These studies also demonstrated that 90–95% of desmin could be sedimented at low speed in the form of filaments after exposure to 5 mM-MgCl₂, pH 7.0. The very rapid absorbance increase during the first 2–5 min with all cations and the absence of a sigmoidal curve shape indicate that either no nucleation step is involved in assembly or, if a nucleation step exists, its half-life is so short that it was not detected. We also cannot exclude the possibility that nuclei may have existed when absorbance measurements were started.

Fig. 2. Electron micrographs of smooth-muscle desmin filaments

(a) A mixture of short filaments and protofilaments is present after 5 min assembly at 2 °C with 5 mM-CaCl₂/20 mM-imidazole/HCl, pH 7.04. (b) Long filaments predominate, but some short and intermediate lengths are also seen after 5 min assembly at 37 °C with 5 mM-CaCl₂/20 mM-imidazole/HCl, pH 7.04. Some filaments have a 20–22 nm axial repeat. Protofilaments are seldom seen in these preparations. (c) Long filaments that are usually tangled characterize desmin samples assembled at 2 °C for 60 min with 5 mM-CaCl₂/20 mM-imidazole/HCl, pH 7.04. When compared with filaments shown in (a), the additional time has resulted in longer filaments. (d) Desmin preparations assembled at 2 °C for 5 min with 150 mM-NaCl/20 mM-imidazole/HCl, pH 7.04, contain many short filaments with irregular or roughened surfaces and occasional long filaments. (e) Long well-formed filaments, which frequently have a 20–22 nm axial periodicity, are present when desmin is assembled at 37 °C for 5 min with 150 mM-NaCl/20 mM-imidazole/HCl, pH 7.04. (f) Long filaments, which seldom have an axial periodicity, are formed when desmin is challenged with 150 mM-NaCl/20 mM-imidazole/HCl, pH 7.04, for 60 min at 2 °C. Length and uniformity of filaments have both increased when compared with (d). Scale bar represents 0.3 µm.

Fig. 3. Absorbance of desmin in the presence of Cu^{2+} at 2, 22 and 37 °C

Desmin was assembled in 2 mM- CuCl_2 /20 mM-imidazole/HCl, pH 7.04. Values from four preparations were averaged.

Although bivalent cations produced higher absorbance values than did the univalent cation, the ionic strength of the 150 mM-NaCl filament-forming buffer is much higher than for solutions containing either 5 mM- CaCl_2 or 5 mM- MgCl_2 . For this reason, we repeated the absorbance measurements (results not shown) in the presence of 15 mM-NaCl buffered with 20 mM-imidazole, pH 7.04, a filament-forming buffer with the same ionic strength as the CaCl_2 or the MgCl_2 solutions. The absorbance values were much lower than those observed with 150 mM-NaCl or with any of the bivalent cations. If 5 mM- MgCl_2 was added to the 15 mM-NaCl solution, the ionic strength increased from 0.035 to 0.05, but the absorbance increased 2–4-fold. This indicates that, even at comparable or identical ionic strengths, bivalent cations are more effective promoters of desmin assembly.

The appearance of negatively stained filaments (Fig. 2) and the association between increased filament length and increased temperature and time (results not shown) indicate that the increased absorbance as time and temperature increased is primarily due to increased filament length. Filament diameters, when averaged over the four physiological cations and the three temperatures (bottom of Table 2), are significantly smaller after 60 min than after 5 min. If these desmin preparations were dialysed overnight, the mean filament diameter was consistently between 10 and 11 nm. The decrease in diameter and the increased uniformity in diameter along a filament as dialysis time increases indicate that a tightening of the structure and more highly ordered packing of subunits are occurring. This suggests that time in dialysis is a variable that must be considered when filament diameters are measured.

The absorbance response obtained with non-physiological cations (Fig. 3) included both higher values than with physiological cations and a gradual decrease in absorbance, typically after 5 min. Negative staining showed that, both in suspension and at the bottom of the cuvette, large masses of filaments were the only structures in these samples. Some of these long tangled filaments were unable to remain suspended in the cuvette. Their accumulation as a small amount of flocculent material at the bottom of the cuvette accounts for the absorbance decrease. Diameters of filaments assembled in the presence of either CuCl_2 or NiCl_2 are larger (mean 15.8 nm) than for those assembled in the presence of the physiological cations (mean 14.3 nm).

These findings demonstrate that Cu^{2+} and Ni^{2+} ions have the opposite effect on desmin to that on microtubules, which are disrupted (Roth & Sigenaka, 1970). Although microtubules are solubilized at lower ionic strengths (Weisenberg, 1972) than for intermediate filaments, Mg^{2+} promotes, but Ca^{2+} inhibits, the assembly of microtubules *in vitro* (Scheele & Borisy, 1979). These results show that intermediate filaments and microtubules respond differently to particular bivalent cations.

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