Reconstitution of Cytokeratin Filaments In Vitro: Further Evidence for the Role of Nonhelical Peptides in Filament Assembly

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ABSTRACT The in vitro renaturation and assembly of cytokeratin molecules to form intermediate filaments (IF) illustrates that these molecules contain all of the structural information necessary for IF information. These molecules contain nine structural domains: the amino- and carboxyterminal extra helical regions, and three conserved extra helical segments that separate four helical rod-like domains. Chymotrypsin treatment of these molecules removes the end-peptide domains and inhibits the self-assembly process.

We have examined the renaturation and assembly of cytokeratin molecules using solution conditions that favor the presence of intermediate forms of IF organization. Dialysis against low salt buffers revealed the presence of bead-like chains of filaments in which the 6–8-nm beads are separated by a distance of 21 nm. These data suggest that a lateral stagger of protofilaments was among the primary events in IF assembly. Chymotrypsin-modified cytokeratin enriched for α-helix barely initiated a turbidity increase at conditions favoring self-assembly. Addition of small amounts of intact cytokeratin accelerated the rate and extent of this reaction. These results indicate that the nonhelical peptides on intact cytokeratin potentiate the assembly of IF by orientating the stagger of laterally associated protofilaments.

Intermediate filaments (IF)[1] (7–11-nm diam) constitute a group of cytoskeletal structures that, on the basis of solubility and immunological properties and tissue and cell of origin, have been classified into five distinct subclasses. These include vimentin of mesenchymal cells, muscle desmin, astrocyte glial fibrillary acidic protein, neurofilaments of neuronal tissues, and keratins or cytokeratins of epithelial cells (1, 2). Although these structures are heterogeneous, they possess several common structural features. Collectively, IF contain extensive α-helical sequences that assume a coiled-coil conformation (3), form morphologically similar intermediate-sized filaments in vivo and in vitro (4), and contain similar peptide domains (3). Recently, amino acid sequence data of all of the IF protein types have been reported (5–11). Compared with the partial sequence of wool microfibrillar keratins (6, 9–11), a significant level of homology has generally been noted to exist. These data confirm previous biochemical and immunological studies advocating a relationship between these groups of proteins upon which structural models for IF were based (13–20).

Partial amino acid sequences have been derived for two wool microfibrillar fragments; the data revealed existence of at least two distinct sequences of microfibrillar keratins (type I and type II) that are only approximately one-third homologous with each other (21, 22). Recently, Hanukoglu, Fuchs, and co-workers (9–11) have demonstrated the existence of two classes of epidermal keratins, each of which shares homology with a type of microfibrillar keratin. The predicted amino acid sequence derived from the cDNA of these later proteins have indicated a molecular basis for variability between type I and II cytokeratins. They have shown that all IF proteins contain a central region with four helical domains that are separated by at least three conserved sites of helix interruptions. In addition, the nonhelical ends of both of types of cytokeratin were shown to contain tandem repeats that distinguish them from other IF and from the microfibrillar keratins (11). Steinert et al. (23) have also reported the amino

[1] *Abbreviations used in this paper*: IF, intermediate filaments.

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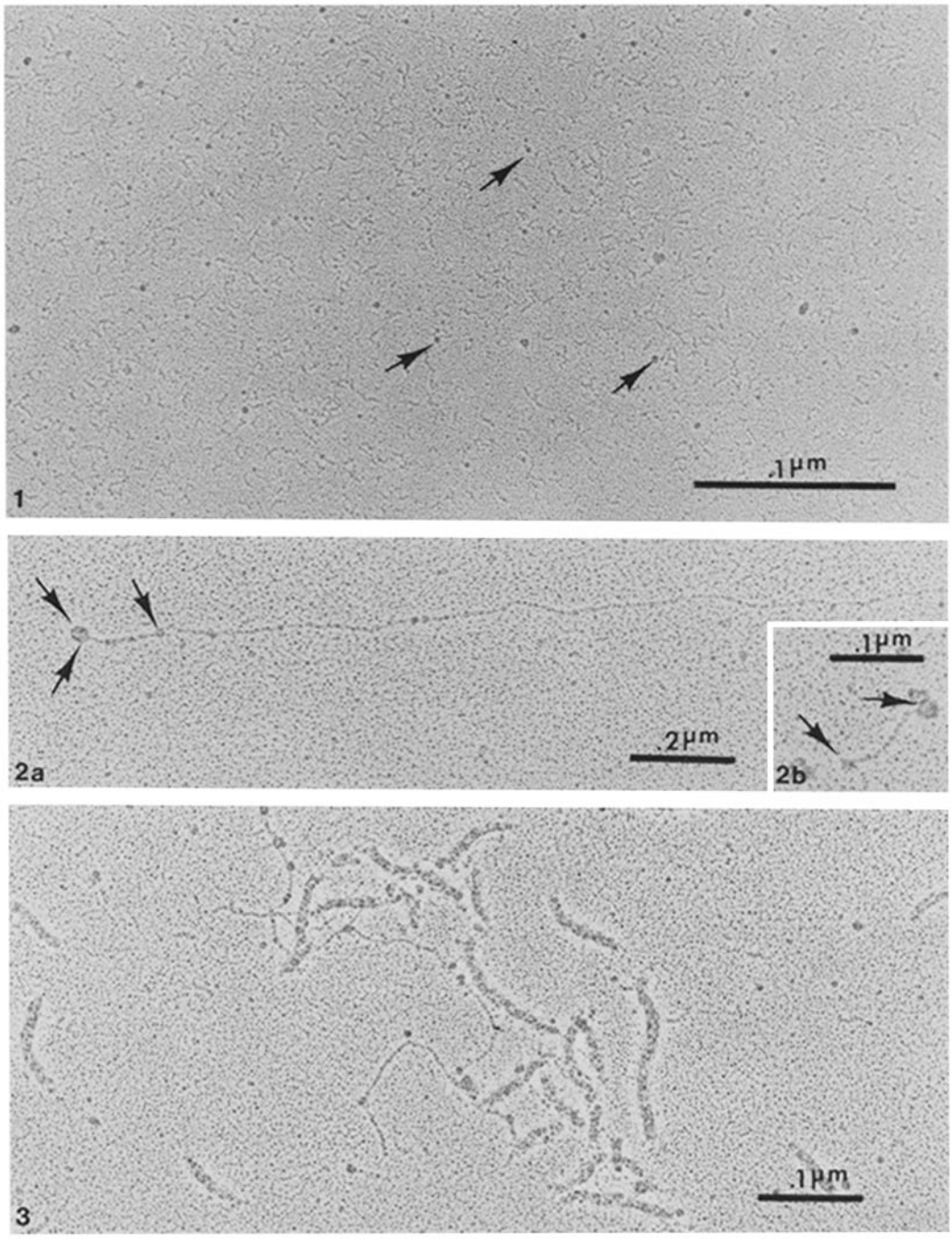
acid sequence for mouse epidermal keratins and have shown that the structural features are essentially similar.

Since denatured monomers of IF show a strong tendency to refold and reassemble into filaments morphologically indistinguishable from native structures (24), it is apparent that all of the information needed for filament assembly is contained in the molecules themselves. A number of studies (12, 25–29) have suggested that the assembly of coiled-coil protofibrils of IF are dependent upon hydrophobic and ionic interactions among the helical domains of the protein subunits. A periodicity in the occurrence of nonpolar residues has been noted in the major helical sequence of wool microfibrillar keratins (25, 27) and similar periodicities have been noted in cytokeratins (10, 11) and in desmin (6). In addition, periodicities of acidic and basic residues in IF and microfibrillar keratins have been predicted using Fourier analysis (12, 26, 28).

Recently, limited proteolytic degradation of IF end-peptides with chymotrypsin was shown to have little effect on filament morphology (23). Analysis of the digestion products from the later preparations revealed that a large part of the total glycine and almost all of the phosphate were removed after 10 min

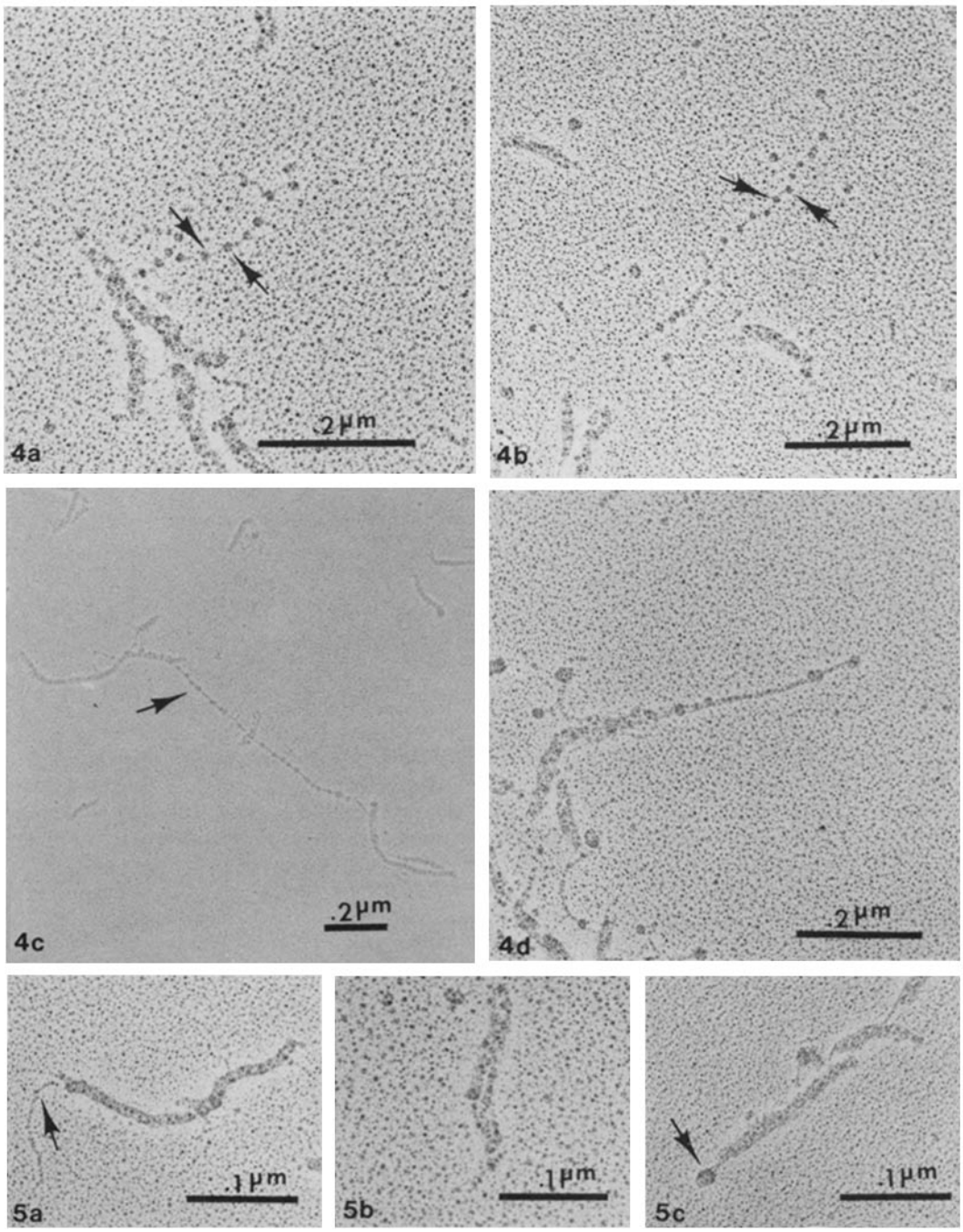


FIGURES 1–3 Fig. 1: Electron micrograph of rotary-shadowed bovine cytokeratin, denatured, and solubilized in 8 M urea after dialysis against low salt buffer containing 4 M urea, 2 h. × 300,000. Fig. 2: Prolonged dialysis of cytokeratin molecules for 12–24 h against 4 M urea in low salt buffer at pH 8.0. (*a*) × 85,000; (*b*) × 150,000. Fig. 3: Rotary shadowed cytokeratin molecules after dialysis against low salt buffers at pH 8.0 for 2 h. × 160,000. Micrographs shows both the presence of protofilaments and short rods. Arrows indicate globular particles associated with short thread-like structures.

of treatment with chymotrypsin. Interpretation of the results suggests that keratin filaments possess a structural organization where the terminal glycine-rich sequences protrude from a conserved core structure into which the coiled-coil helical segments are packed (23). Although it is generally acknowledged that the lack of homology among the non-α-helical sequences of IF subunits contributes to the observed variations in antigenicity and solubility (2, 18), and that these regions are involved in an end-to-end linkage of IF proteins (15, 17, 20), the role of these terminal end-sequences in assembly has not been defined. In the present work, we provide evidence demonstrating an obligatory role for the nonhelical, chymotrypsin-sensitive, end peptides of cytokeratin in IF assembly. Specifically, we indicate that the nonhelical terminal peptides probably orient the stagger of protofilament molecules that facilitates an interaction between adjacent helices. This event is suggested to be an early step in IF assembly.

MATERIALS AND METHODS

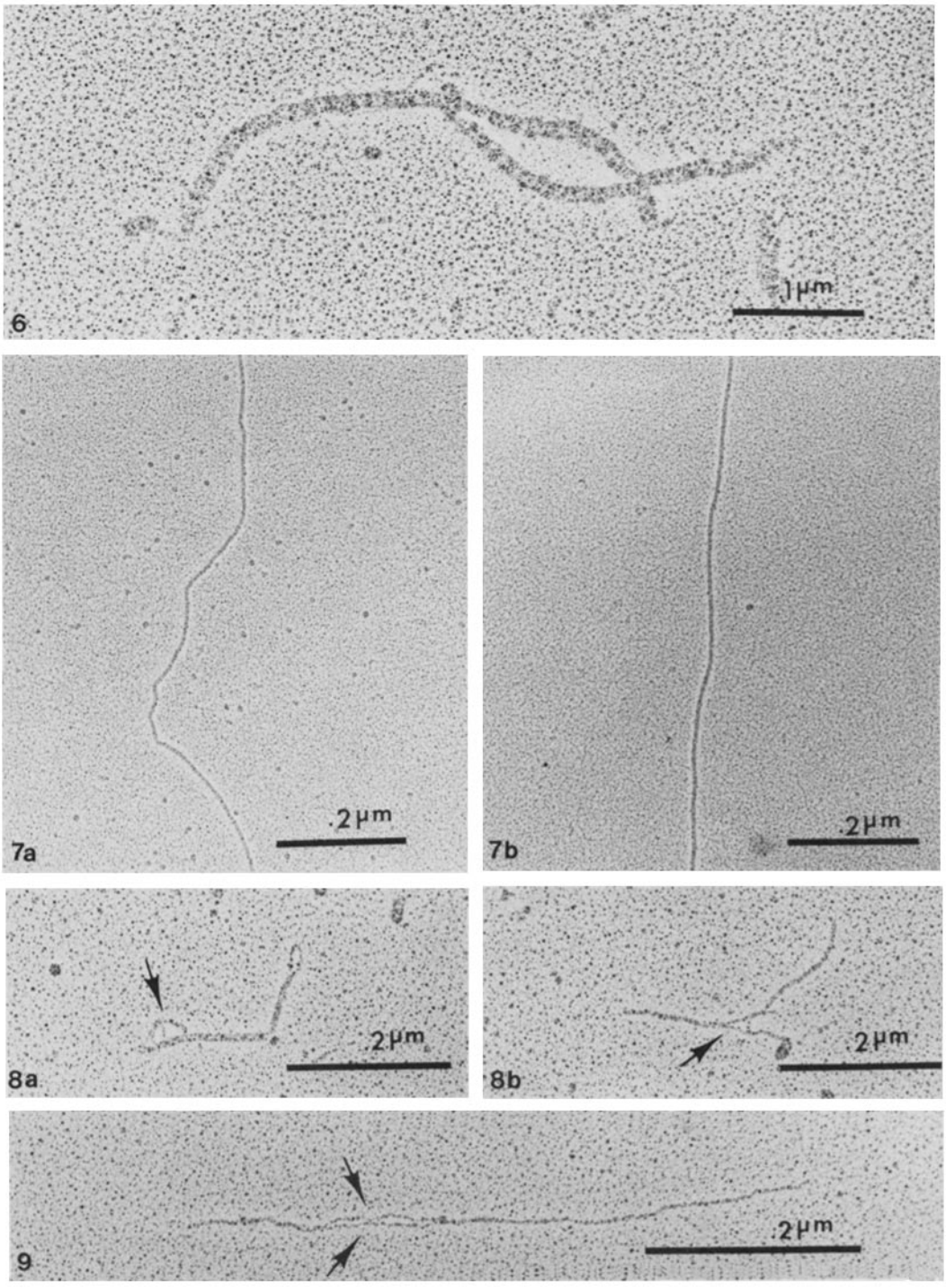
Cytokeratin Preparation: Cytokeratin was prepared from bovine snout epidermis by a modified method described by Franke et al. (24). Briefly,



FIGURES 4 and 5 Fig. 4: Rotary-shadowed cytokeratin molecules after dialysis against low (*a–d*) salt buffer at pH 8.0 for 1–3 h. (*a* and *b*) Beaded filaments with a distance between (6–8 nm) beads (arrows) of 21 nm; (*c*) beaded filaments (arrows) joining two short rod filaments; (*d*) later stages of filament assembly showing rods and condensed protofilaments with occasional globular beads (6–8 nm). (*a* and *b*) × 70,000; (*c*) × 50,000; (*d*) × 100,000. Fig. 5: Rotary-shadowed short rod forms of cytokeratin containing (*a*) frayed end-regions (arrow) composed of protofilaments; (*b*) coiled filaments; and (*c*) annular structures (25 nm). (*a–c*) × 170,000.

the epidermis was homogenized in distilled water made pH 9 with NaOH. The homogenate was centrifuged at 750 *g* and surface debris and pellet were discarded. The supernatant was further centrifuged at 4,000 *g* for 30 min and the resulting pellet was suspended in 2 mM $NaHCO_3$ (pH 9.0), 2 mM mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride. The suspension was centrifuged at 100,000 *g* for 30 min. The samples were then exposed to chymotrypsin (0.5 $\mu g\ ml^{-1}$, 0.1% [wt/wt] for periods of 5 and 10 min) after methods of Steinert et al. (23). After digestion the filaments were pelleted by centrifugation. For reconstitution experiments, subsequent to digestion, the filaments were redissolved in 8 M urea buffer and centrifuged as before. The extent of the α-helix in the samples was determined by circular dichroism using a Jasco J41-C automatic spectropolarimeter (Jasco Inc., Easton, MD) equipped with a quartz cell of 1-cm path length (30).

Reconstitution of Cytokeratin Filaments: Cytokeratin filaments were reconstituted from urea-denatured monomers as previously described (31, 32). These procedures were in some instances modified after those described by Franke et al. (24), to enrich for intermediate stages of filament formation during reconstitution from denatured monomers. For these studies, low salt buffers containing 1 mM Tris-HCl (pH 8.0), 10 mM mercaptoethanol, and 4 M urea were employed. In other instances, samples were dialyzed against 5 or 10 mM Tris HCl (pH 7.6). The quantitative assessment of filament assembly was accomplished by turbidimetric analysis of the light-scattering properties,



FIGURES 6–9 Fig. 6: Long intermediate-sized filaments observed under conditions used in Fig. 5. × 200,000. Fig. 7: Dialysis of cytokeratin solutions against 10 mM Tris buffer at pH 8.0; (*b*) revealing long compact intermediate-sized filaments. (*a*) IF treated with chymotrypsin for 5 min. (*a* and *b*) × 100,000. Fig. 8: IF of cytokeratin treated for 10 min with chymotrypsin. (*a* and *b*) × 125,000. Fig. 9: Rotary-shadowed IF of cytokeratin treated with chymotrypsin for 10 min. × 165,000. Arrows indicate regions of fraying after enzyme treatment.

from in vitro reconstitutions monitored at 300 nm, in a Beckman spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA) (33).

Electron Microscopy: Solutions of filamentous cytokeratin was mixed with glycerol to 60% and sprayed on freshly cleaved mica as described by Kühn et al. (34). Shadowing was performed with carbon/platinum (95%/5%) at an angle of 6° and stabilized by coating with carbon at an angle of 90°. Electron micrographs were taken in a Phillips 201 electron microscope. Magnifications were corrected as previously reported (34).

RESULTS

Cytokeratin solutions were dialyzed against low salt, 4 M urea buffers to favor the presence of intermediate forms of IF organization during renaturation and assembly. Dialysis for periods of 1–3 h produced thin, short thread-like structures that were associated with globular particles (Fig. 1). Continued dialysis of these solutions resulted in protofilaments that ranged in length from 25 nm to 1 μm (Fig. 2). These structures contained both globular structures, 6–8-nm regions, and annular structures, 18–25-nm diam (Fig. 2).

To accelerate these conformational changes and assembly reactions, cytokeratin solutions were also dialyzed against low salt buffers lacking urea (1 mM Tris, pH 8.0). Reconstitution by dialysis against these buffers for 10–30 min resulted in structures similar to those observed with urea shown in Figs. 1 and 2. However, continued dialysis under these conditions (1–3 h) produced short, rod-like filaments (Fig. 3–5) with diameters typical of intermediate-sized filaments (1). During early periods, (1 h), protofilaments (2.5–3.5 nm diam) were seen in close association with these short rod forms (Figs. 3 and 4). The protofilaments often possessed 6–8-nm globular regions ordered in a period of ~21 nm (Fig. 4, *a–c*). After longer intervals of dialysis, protofilaments were less apparent (Figs. 4*d* and 5), although occasional frayed end-regions (Fig. 5*a*) could be observed on the rod forms. Some of these short, rod-like filaments were loosely packed and demonstrated coiling of substructures (Fig. 5*b*). Annular structures of 18–25 nm were also seen, usually at one end of some of the short rods (Fig. 5*c*). Long, intermediate-sized filaments were uncommon. However, when noted, these structures exhibited an axial period of ~20 ± 1.5 nm (Fig. 6). Dialysis of the cytokeratin solutions against 10 mM Tris buffers, pH 8.0, resulted in further polymerization. The resultant filaments were compact (8–14 nm diam), long structures that ranged up to 2 μm long (Fig. 7*b*).

Cytokeratin filaments were treated with chymotrypsin to remove nonhelical peptides. These procedures were performed to study the behavior of α-helical-enriched structures at conditions favoring renaturation and reassembly. Untreated filaments were noted to contain 38 ± 5% helical material whereas 5 min of treatment with chymotrypsin enriched this content to 58 ± 5%. The filaments isolated after limited proteolysis appeared morphologically similar to untreated structures (Fig. 7*a*). 10 min of treatment with chymotrypsin further increased the α-helical content to 67 ± 4%. Although similar to untreated structures, the filaments were often shortened, frayed (Fig. 8), and extremely thin, averaging 2.5–4.5 nm diam (Fig. 9).

After chymotrypsin treatments, the isolated filaments were made soluble in 8 M urea buffers by being heated to 50°C for 10 min. Reconstitution of these polypeptides were kinetically evaluated by monitoring the changes in turbidity (light scattering) at 300 nm (18). Samples were taken at various periods of filament assembly and examined by electron microscopy as before. The kinetics of assembly of the filaments are depicted in Fig. 10. At polypeptide concentrations of 0.65 mg/ml, untreated cytokeratin solutions produced a sigmoidal turbidity curve. These curves were characterized by an initial lag period followed by a more rapid increase in turbidity.

Examination of samples taken during the early periods of turbidity increase (30–40 min) revealed thin, thread-like structures reminiscent of the intermediate structures formed by dialysis against low salt buffers (Fig. 1). Samples taken at 100–120 min contained both thin, thread-like structures and protofilaments similar to those observed in Fig. 2. Later samples (150 min) contained both short rod forms and protofilaments that were identical to the filaments seen after dialysis (1–3 h) against low salt buffers lacking urea (Figs. 3–5). The turbidity plateau contained both short rod forms and long (2 μm) IF.

Reconstitution of cytokeratin filaments treated for 5 min with chymotrypsin lengthened the lag period before development of turbidity. This was followed by an increase in turbidity culminating in an optical density that was only 25% of the final turbidity observed with untreated cytokeratin solutions (Fig. 10*b*). 10 min of treatment further decreased the extent of final turbidity and prolonged the lag period (Fig. 10*C*). Examination by electron microscopy of the final products of the latter samples revealed only aggregated thread-like intermediate structures (Fig. 11).

Reconstitution mixing experiments were performed between α-helical-enriched polypeptides isolated after 10 min of treatment with chymotrypsin and untreated, denatured cytokeratin solutions. Various volumes of cytokeratin polypeptides treated for 10 min with chymotrypsin and intact cytokeratin solution at a concentration of 0.65 mg/ml were mixed and monitored for increases in turbidity. The α-helical domain content was kept constant. In these experiments the lag periods of the chymotrypsin-treated cytokeratin/intact cyto-

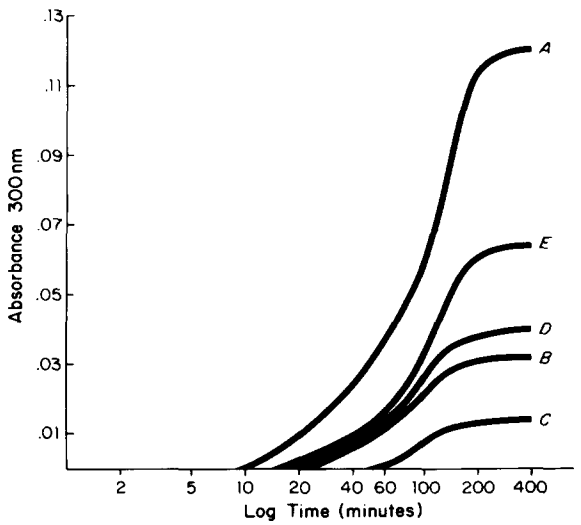
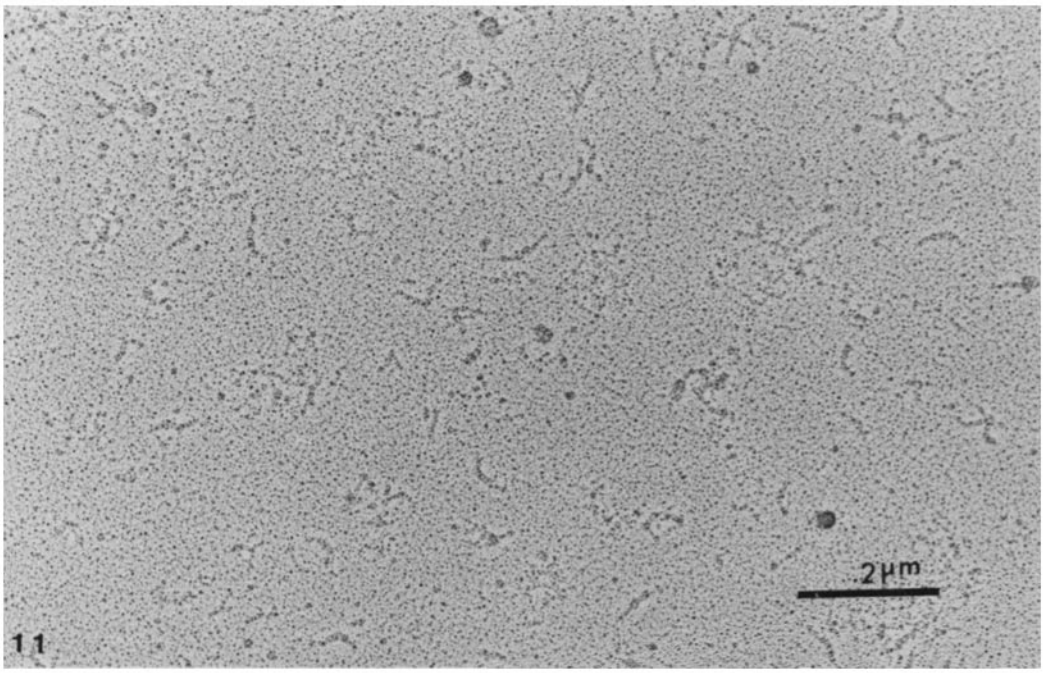


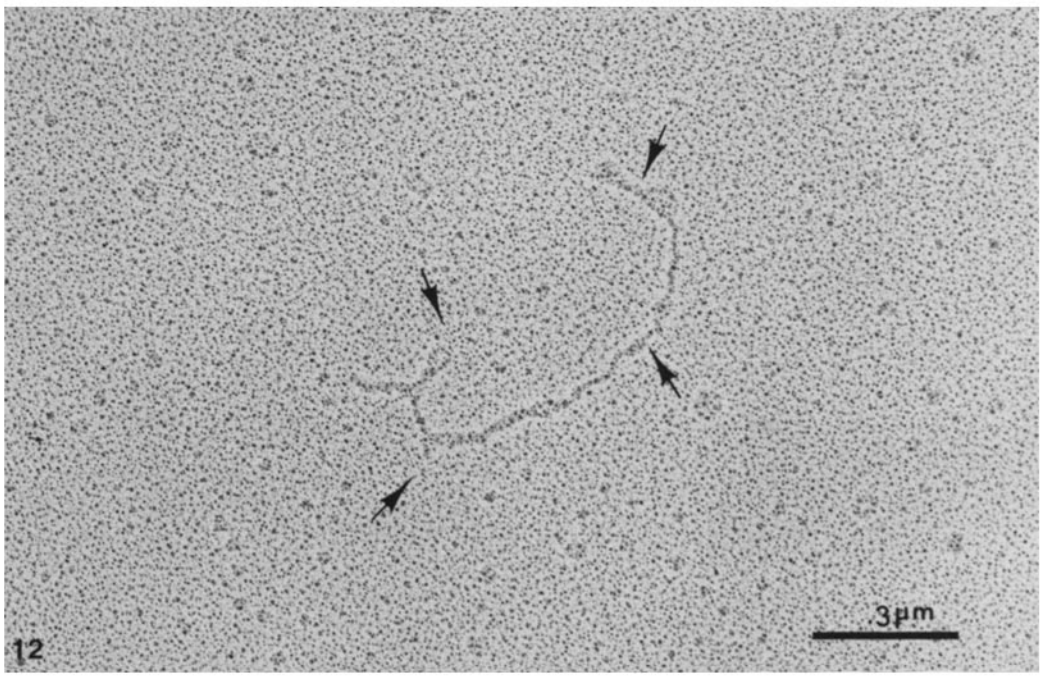
FIGURE 10 The effect of chymotrypsin treatment of IF and the addition of intact cytokeratin molecules to chymotrypsin-treated cytokeratin molecules on the rate of development of turbidity. The concentration of protein was 0.65 mg/ml or at an α-helix content equal to that in a 0.65 mg/ml solution of intact cytokeratin. (*A*) Intact cytokeratin solubilized in urea and heated to 50°C for 10 min. (*B*) Cytokeratin filaments treated for 5 min with chymotrypsin before isolation, solubilization, and denaturation. (*C*) Cytokeratin treated for 10 min with chymotrypsin prior to solubilization and denaturation. (*D*) A 95% solution of 10-min chymotrypsin-treated cytokeratin to which 5% intact cytokeratin has been added before renaturation. (*E*) A 90% solution of 10-min chymotrypsin-treated cytokeratin to which 10% intact cytokeratin has been added.

keratin system were shortened with increasing addition of intact cytokeratin molecules (Fig. 10, *E* and *D*). After 24 h of incubation, electron microscopic examination of the final products of assembly revealed the presence of thread-like structures and anomalous, ribbon-shaped structures that lacked the ordered structure of ideally formed IF formed from intact cytokeratin solutions (Fig. 12). Fig. 13 shows the effect of chymotrypsin digestion on intact cytokeratin IF.

DISCUSSION

Lateral association of protofilaments has been previously recognized (24). Results of the present study demonstrate that lateral associations are among the primary events in IF assembly and demonstrates that they are directed by the nonhelical end-peptides of IF. During the formation of IF, before the development of smoothly contoured protofilament threads, the structures are located at one pole of the protofilament subunit (Figs. 4 *a* and 7 *d* of reference 24). Therefore, it appears that during renaturation the terminal, nonhelical peptides, which are highly convoluted flexible structures, assume a globular form rather than existing as extended threads (Fig. 4 *a*). This seems particularly true for the larger, carboxyterminal polypeptides. Thus, during early stages of filament





FIGURES 11 and 12 Fig. 11: Rotary-shadowed cytokeratin molecules treated with chymotrypsin for 10 min, denatured, and solubilized in 8 M urea followed by dialysis against 10 mM Tris for 8 h. × 100,000. Fig. 12: Rotary-shadowed cytokeratin molecules (arrows) that were first treated with chymotrypsin for 10 min, denatured, and solubilized in 8 M urea then mixed with denatured solubilized intact cytokeratin (10%) and dialyzed against 10 mM Tris for 24 h. × 67,000.

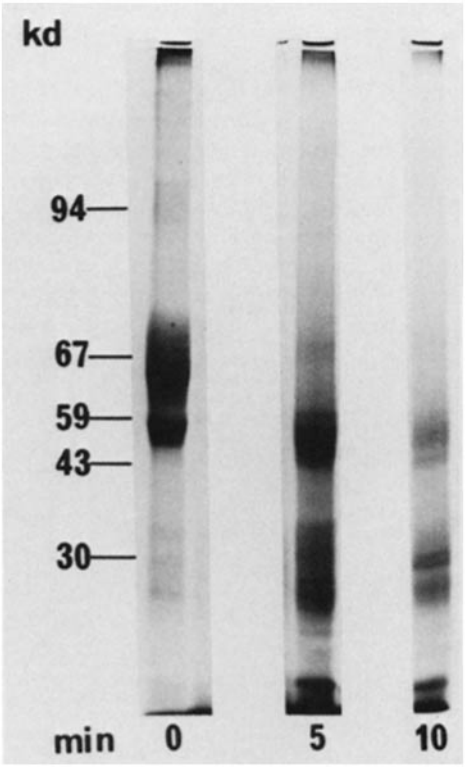


FIGURE 13 PAGE (23) of chymotrypsin digestion products of bovine epidermal cytokeratin.

assembly, the amino- or carboxyterminal, nonhelical regions are seen to interact in a relationship with adjacent molecules to form an extended, beaded structure. Subsequently, a conformational rearrangement must occur such that smooth protofilament threads are formed.

Earlier studies (13, 15, 18, 20, 25) have indicated that proteins that form IF or microfibrillar filaments contain two helical domains of 15–22 nm and that the axial periods of 21 nm observed in the ultrastructure of the filaments formed from these proteins reflect the unit length of the helical domains within each IF protein (35–37). However, more recent models based on sequence data indicate that these proteins are actually formed from four centrally located helical domains that are separated from each other by three conserved, nonhelical regions (10, 11). Although it still is not clear whether these conserved nonhelical regions reverse the direction, or cause a loop in the polypeptide chain at these points, their small size and probable structure suggests that they contribute little, if anything, to the overall lengths of the coiled-coil α-helical region. Conservative estimates, based on the assumption that a single residue is equal to ~0.2 nm, can account for only ~3 nm of additional length in the 59,000-mol-wt mouse epidermal keratin (23). This latter dimension approaches the resolution of shadowing techniques used in this study. Thus, the period of 21 nm between globular regions observed in the intermediates of IF formation can only be accounted for by placing the interaction site of one of the end peptides between laterally associated coils on an α-helical region, or on one of the conserved, nonhelical loop regions.

The presence of acidic and basic residues with periodicities of 28/3 and 28/10 in the helical regions of IF have been predicted from Fourier analysis (12, 23, 26, 28). These charged residues have been shown to occupy superficial sites on the coiled-coils and have been suggested to play a significant role in the lateral association of neighboring coiled-coils in the IF structure (23). Although sequence data appears to favor the parallel packing of neighboring coils in approximate alignment with the filament axis (23), this model is not in accord with the morphology of intermediate structures observed during early stages of IF assembly in the present study. Our data implies the existence of a systemic axial stagger of laterally associated filaments where neighboring coiled-coils are arranged in a parallel or antiparallel relationship.

Formation of ribbon-like filaments from the 38,000-mol-wt domain of desmin (pH 5.5) and the demonstration that small amounts of trypsin added to solutions of keratin during reconstitution yield paracrystalline arrays with a 5.4-nm axial repeat also support the presence of some degree of axial stagger between helical subunits of IF (20, 38) since the elongation of α-helical segments lacking end-domain peptides can not be seen to assemble in an end-to-end manner. The addition of intact cytokeratin to chymotrypsin-treated cytokeratin solutions potentiated these reactions in the present study, since the lag period for the initial turbidity increase is shortened and ribbons of imperfectly formed filaments are subsequently produced. The formation of ribbons in mixing experiments reported here did not exactly parallel those described in desmin (20). However, the assumption that intact cytokeratin was first incorporated into filaments that are then laterally associated with chymotrypsin-treated polypeptides to form ribbons is untenable. In control experiments dilution of intact cytokeratin to the levels added in mixing experiments was below the critical concentration for filament assembly. These results indicate that nonhelical peptides on intact cytokeratin exert a seeding effect whereby they potentiate the stagger of laterally associated protofilaments by orientating adjacent polypeptides. Nelson and Traub (39) have recently shown that removal of the amino-terminal domain of desmin and vimentin resulted in solutions that would not form IF. Extending their data to the work presented here would suggest that the amino-terminal end-peptides are key in directing the events of assembly.

The role of nonhelical telopeptides in the lateral assembly of protein polymers is not unique. Helseth and Veis (40) have also proposed and tested a model whereby the amino-telopeptide of type I collagen is involved in an interaction with a helical receptor site as a step in the nucleation of collagen during fibrillogenesis. Although the present report supports the recent work of Franke et al. (41), that suggests that certain cytokeratin polypeptides are complimentary and contain sequences that direct their association into specific complexes forming IF subunits, studies are now in progress that will elaborate the role of specific amino- and carboxyterminal peptides of cytokeratin in the regulation of these early interprotofilament associations during renaturation and assembly of IF.

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