Isolation of Intermediate Filament Assemblies from Human Hair Follicles

LESLEY N. JONES* and F. MICHAEL POPE*
*Commonwealth Scientific and Industrial Research Organization Division of Protein Chemistry, Parkville, 3052, Victoria, Australia; and *Dermatology Research Group, Clinical Research Centre (Medical Research Council), Harrow HA1 3UJ, England

ABSTRACT We used developing human hair follicle cells for the isolation of hard α-keratin structural components. Intracellular dispersions examined by electron microscopy contained both individual α-keratin filaments and the tactoid-like filament assemblies observed in situ organized along subfibrillar arms of macrofibrils. The assemblies of average width 47 nm were composed of closely packed α-keratin filaments and originated from the initial filament arrays observed in sections of developing mammalian hair follicles. We have distinguished two types of assemblies: the para-like or hexagonally packed and the ortho-like spiral or whorl type. Axial banding extended across the width of filament assemblies, which suggested that hard α-keratin filaments pack in lateral register and form a lattice that contains interfilamentous bridges. We observed axial banding patterns with periods ranging from 20 to 22 nm, consistent with the 22-nm periodic structure deduced from x-ray diffraction studies and present in models proposed for hard α-keratin and other intermediate filaments. Preliminary biochemical studies of filaments and filament assemblies indicate that they consist of the closely related group of proteins (low-sulfur proteins) ubiquitous among extracts of hard mammalian keratins. Isolated hard α-keratin filament assemblies provide a new and valuable structural entity for investigating the assembly mechanisms involved in the formation of the filament-matrix framework found in hard mammalian keratin appendages.

Specialized mammalian appendages such as hairs, nails, and claws contain keratin proteins packed into a complex filament-matrix intracellular composite (1). The filaments, often termed microfibrils, give rise to an α-type x-ray diffraction pattern and are grouped with that class of 7-10 nm diameter filaments known as intermediate filaments (IFs) found as ubiquitous structural components of eukaryotic cells (2). Five groups of IFs are normally distinguished, the keratin group being least typical with respect to complexity and assembly mechanisms (3). α-Keratin filaments occur as the major structural component in epidermal tissues (soft keratins) and in specialized appendages (hard keratins) where they are associated with a matrix protein phase. Despite many similarities between hard and soft α-keratin filaments, significant differences exist in their protein components (4). α-Keratin filaments consist of several families of closely related protein components and a combination of components from different families is apparently required for their formation (3, 5, 6). Hence, the structural unit in α-keratin is a heteropolymer which has been suggested to be formed from three (3, 7) or four (8, 9) of these component proteins whose helical domains interact to form coiled coils. The components also contain nonhelical regions at both the amino and carboxyl termini and at various intervals along the molecular chains (10).

IFs are usually obtained for study by molecular reassembly of extracted components in vitro (2), but their correspondence to the native structure must be assumed. Methods employing cell lysis of dissected hair follicles have enabled successful isolation of native hard α-keratin filaments (11). Negatively stained filaments isolated by this method were shown to have an internal structure (12), consistent with earlier evidence for a ring-core substructure (13, 14). However, detail of molecular organization was not evident (12) and has not been demonstrated in negatively stained preparations of reconstituted epidermal filaments (5, 15, 16) or other IFs (15, 16). It has

1 Abbreviations used in this paper: IF, intermediate filament; KPT, potassium phosphotungstate; SCM, S-carboxymethyl derivatives.

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proven possible to observe an axial periodicity in metal-shadowed preparations of epidermal and neurofilaments (15, 16) and desmin filaments (15). Periodicities in these filaments ranged from 20 to 22 nm and were in agreement with a 22-nm repeating structure envisaged in models of hard α-keratin filaments based on x-ray diffraction studies (17).

In the present studies we have used modified conditions for the isolation of native hard α-keratin filaments. We have obtained individual filaments and tactoid-like filament assemblies and used them for structural and preliminary biochemical studies. These filament assemblies have proved to be useful structures for observing both intrafilament molecular packing and the interfilament assembly mechanisms involved in the formation of hair.

MATERIALS AND METHODS

Isolation of Native α-Keratin Structural Components: Human hairs were plucked from the scalp of volunteers, and hairs with follicles in the anagen growth phase were selected for subsequent dissection (11) (see Fig. 1). Follicles were immediately immersed in a high antibiotic tissue culture medium at 0°C (solution A). 100 ml of medium contains gentamycin 50 μg/ml, polymyxin B 50 μg/ml fetal calf serum (8 ml), n-glutamine 200 mM/ml (1 ml), and Dulbecco’s modified Eagle’s medium to 100 ml.

Dissection of follicles was undertaken in solution A with the aid of a binocular microscope at ~25×. A polarizing filter was fitted to the microscope to aid in complete removal of inner root sheath layers from presumptive hair shaft cells. Dissected follicles were separated from the hair shaft (Fig. 1) and transferred into a 1-ml capped sterile centrifuge tube (Eppendorf) containing solution A at 0°C. About 30 follicles were collected under these semisterile conditions to minimize bacterial contamination. The dissected follicles were washed twice by gentle centrifugation and washed twice with Pack’s solution (100 ml contains glucose 0.1 g, NaCl 0.88 g, and KCl 0.04 g, and sterilized first by filtration through a sterile 0.22-μm disposable Millipore filter [Millipore (U.K.) Ltd.] and then once with a 0.85% wt/vol sterile saline solution. This was followed by a single brief washing with sterile distilled water, and the follicles were pelleted as above. The excess water was carefully removed, leaving only a minimal amount covering the pelleted follicles. They were then left for a few minutes to take up water, resulting in an observable swelling. A sterile glass rod was used to gently break up the follicles and then the final volume of water was increased to 300 μl.

Electron Microscopy: Carbon-Formvar coated grids were glow-discharged and usually rinsed in n-hexane containing 0.015% wt/vol cetyl alcohol and dried. A drop of the follicle dispersion obtained above was placed onto the grid, left until almost dry, and negatively stained with 0.3% wt/vol aqueous uranyl formate in a freshly prepared 2% wt/vol aqueous solution of potassium phosphotungstate (KPT) at pH 7.2 (12). In certain cases negative staining was followed by a wash with n-hexane containing 0.015% wt/vol cetyl alcohol (18). Specimens prepared for unidirectional shadowing were placed on agar gels to reduce salt concentrations (19), and grids were then shadowed with carbon-platinum at angles of 70 and 75°.

Scalp biopsies obtained by surgery were fixed initially overnight in 0.1 M phosphate-buffered 2.5% vol/vol glutaraldehyde containing 2 mM MgCl2, washed three times for 15-min intervals in 0.1 M phosphate buffer containing 2 mM MgCl2, and postfixed for 2-3 h in 0.1 M phosphate-buffered 1% vol/wt osmium tetroxide. They were then washed three times with distilled H2O, immersed in 0.5% wt/vol aqueous uranyl acetate, left overnight, and rinsed in distilled H2O. Dehydration was through a graded series of alcohols followed by immersion in propylene oxide. Pre-embedding was performed in a 30:50 mixture of propylene oxide and Epon. The subsequent embedding in Epon at 37°C overnight was followed by polymerization at 60°C.

Specimens were examined in A.E.I. 801, Phillips EM 300, and Hitachi HS-8 electron microscopes.

Biochemical Studies: Approximately 40 human anagen follicles were plucked, dissected, and used for preparation of filament and filament assembly dispersions as described above. In addition, follicles were dissected free of surrounding inner and outer root sheath cells and retained as controls. The dispersions were purified by a centrifugation procedure initially at 3,000 g for 10 min at 4°C. The supernatant was collected and centrifuged at 105,000 g for 1 h at 4°C to yield a pellet. This pellet and follicle controls were extracted with a solution containing 5 M urea, 0.05 M dithiothreitol, 0.25 M Tris, and 0.01 M EDTA, pH 9.5, for 3 h at 20°C. Extracts were converted to S-carboxymethyl derivatives (SCM) using [2-35S]iodoacetic acid. Initial electrophoresis of the SCM-filament pellet and SCM-follicle extract on 10% wt/vol polyacrylamide gel disks in the presence of 8 M urea at pH 8.9 was followed by a second-dimensional separation on polyacrylamide gel slabs (10% wt/vol) in the presence of SDS. Radiolaabeled proteins were located by fluorography on x-ray film and exposed for periods at ~80°C.

RESULTS

Structural Studies

Cells used for the isolation of hard α-keratin filaments and filament assemblies were derived from around the region indicated by the arrow in Fig. 1A. In this longitudinal section of a human anagen hair follicle germinal cells undergo elongation and synthesize keratin proteins in the lower cortical zone of the developing hair shaft. The cell cluster shown in Fig. 1B is typical of the products obtained following the procedure described in the previous section of this paper. Careful dissection has eliminated the possibility of contamination from inner and/or outer root sheath cells. A preparation of the dispersion products obtained after lysis of isolated presumptive cortical cells is presented in Fig. 2, A and B. These dispersions, negatively stained with a neutral solution of KPT, show fibrillar structures (macrofibrils) made up of individual α-keratin filaments aligned roughly parallel to the longitudinal axis of the fiber. The macrofibril shown in Fig. 2A contains clearly delineated structures with a rectangular appearance. These lightly staining rectangular structures show generally uniform dimensions and alternate with darker staining regions along the subfibrillar arms that compose the macrofibrils. Dispersive effects encountered during cell lysis and specimen preparation for electron microscopy probably disrupted any observable regular organization of filament assemblies within subfibrillar arms. Negative staining of macrofibrils with uranyl salts including uranyl acetate and uranyl formate caused increased dispersion of filaments and fully disrupted the distribution of the rectangular structures. Closer examination of the lightly staining rectangles after negative staining with KPT and uranyl salts revealed them to be composed of closely packed α-keratin filaments (filament assemblies) that exhibited a high resistance to penetration by stain. In longitudinal sections obtained from the lower regions of human hair follicles, we observed filament assemblies distributed within macrofibrils as darkly staining rectangles (Fig. 4B). Although positive staining has not proved useful for observing ultrastructural details, the demonstration of these assemblies in situ has served to eliminate the possibility that structural artifacts were introduced as a result of the isolation or negative staining procedures. The lateral widths of filament assemblies calculated from micrographs fell into two classes of widths. The first, comprising the bulk of assemblies contained 30 observations (from Fig. 2B), had a width of 47 ± 2.4 nm mean ± SD, and the remainder consisted of six observations with a width of 80 ± 5.1 nm mean ± SD. The latter class probably contains lateral aggregates of two filament assemblies. Greater variation existed in lengths which were usually in the range 100–200 nm.

Filament assemblies studied at high resolution after negative staining with uranyl formate and neutral KPT are presented in Fig. 3. When the filament assembly was stained with uranyl formate (Fig. 3, A and B) its integrity was apparently retained, but the frayed filamentous ends or crucible appearance of the assemblies indicated a potentially degradative
Fig. 1 (A) Longitudinal section through a human hair follicle (anagen phase) stained with 2% wt/vol toluidine blue. The base of the follicle is in contact with the dermal papilla, and columns of developing epithelial cells that comprise the hair cortex (H. CO) and cuticle (H. CU) originate from this region. Also, cell columns of the inner root sheath including the cuticle (IRSC), Huxley's layer (HU), and Henle's layer (HE) appear to be emerging from germin al epithelia in contact with the dermal papilla. The intensely dark regions are accumulations of melanin granules found in melanocytes and cortical cells. x 200. (B) A phase-contrast micrograph shows a cluster of developing cortical cells undergoing elongation which were isolated from the lower region of the hair follicle (arrow) in A. Cortical cells in the early stages of maturation were dissected from human hair follicles and used to isolate keratin components. x 700.

Effect presumably caused by the acidic pH of this stain solution. Substructure within individual filaments and filament assemblies was not easily observed after negative staining with uranium salts. Longitudinal striations resulting from closely packed filaments within the assembly can be seen in Fig. 3, A and B. Filament packing was observed as either parallel to the longitudinal axis of the assembly or inclined at a small angle ranging from 7 to 10° with this axis. In the latter case we determined the angles of inclination from direct measurements of micrographs containing this structural feature (an angle of 8.6 ± 1.0° mean ± SD was calculated from 16 observations). The delineation of filaments packed longitudinally was more clearly observed in assemblies stained with neutral KPT (Fig. 3, C and D) and appeared as fine lines of deposited metal aligned parallel to the longitudinal axis in Fig. 3C and inclined with this axis in Fig. 3D. The close packing of filaments possibly occurs as a result of collapse after drying and/or chemical attraction of protein components and has probably deterred the penetration by heavy metals into the assemblies.

If the filament assemblies were considered as cylindrical arrays, the inclined assemblies observed in Figs. 3, B and D (assuming an average angle θ 8.6°) would contain filaments following a helical path around the surface of this cylinder. The period generated by the helix repeating over an extended length of assembled filaments (20) of mean width 47 nm would be given by $(\pi \times \text{average diameter}/\tan \theta) = (47 \pi / \tan 8.6) = 977 \text{ nm}$.

Transverse sections through the upper-bulb regions of embedded human hair follicles (Fig. 4A) have demonstrated the presence of two different cell types based on the relative stain uptake of their respective cytoplasms. The macrofibrils contained by the more lightly stained cells were observed to be composed of filaments which were less tightly packed than those in the cells with a densely stained cytoplasm. The less tightly packed macrofibrils are analogous to well-defined structures observed in the para-cortical cells of fine wool fibers. Similarly, the more densely stained, closely packed macrofibrils resemble the structures found in ortho-cortical cells. Hence, the terms para-like and ortho-like have been used to describe these structures. The para-like macrofibrils contain hexagonally packed filament assemblies and the ortho-like macrofibrils, the helically packed filament assemblies (1, 27, 28).

Unidirectional shadowing using platinum-carbon demonstrated an axial periodicity in individual filaments and inter-
FIGURE 2 Dispersion products obtained after lysis of dissected cortical cells showing macrofibrils and their component keratin filaments together with lightly staining rectangular filament assemblies. The dimensions of the macrofibril shown in A are $12 \times 1.7 \mu m$. Individual keratin filaments (F) are clearly visible (B) and these can often be seen packing closely as lateral assemblies in the lightly staining rectangles. Two populations of filament assemblies were determined but most belong to a class of mean width 47 nm. Specimens were negatively stained with 2% wt/vol potassium phosphotungstate, pH 7.0. Bars, 1 \mu m. x 25,000.

Axial banding patterns were observed in filament assemblies (see Figs. 5, A–D). The micrographs in Figs. 5, A–C show individual hard $\alpha$-keratin filaments with periodicities evident at intervals within each filament. In shadowed filament assemblies (Fig. 5D) evidence for longitudinally aligned filaments supports previous observations and serves to eliminate the possibility of staining artifacts. Shadowed filament assemblies displayed dimensions similar to those of negatively stained preparations.

Preliminary Biochemical Studies

Protein compositions obtained by two-dimensional PAGE of the SCM-filament pellet and SCM-follicle extract are presented in Fig. 7. In previous studies fractionation of keratin extracts indicated the existence of two major classes of proteins. These have been extensively characterized (1) and denoted low sulfur and high sulfur according to their relative cysteine contents. The respective protein classes are labeled assemblies that showed axial banding to eliminate the possibility that individual filaments lay across assemblies at regular spacings. However the probability of this occurring over even a few repeats is very low. Average spacings in axially banded filament assemblies obtained from various images were in the range 20–22 nm.

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L.S.P and H.S.P in the follicle extract control (Fig. 7A). Electrophoretic patterns obtained from SCM-filament pellets (see Fig. 7B) contain low-sulfur proteins and have not shown any evidence for presence of high-sulfur or other intermediate filament protein components. Extended exposures of labeled extracts showed no evidence for the existence of high-sulfur proteins. Low molecular weight material of unknown composition was present in extracts of both control follicle and pellet.

**DISCUSSION**

The complexity of proteins contained within α-keratin filaments distinguishes them from other IFs (2) and probably plays a role in the function and specialization of hard mammalian keratin appendages. Although differences between molecular chains have been shown to be obligatory for reassembly of epidermal filaments (5) it is not known if these same requirements apply to hard α-keratin filaments. The filaments of hard and soft keratins are morphologically similar, but a recent publication on intermediate filaments proposed that the significant chemical differences warrant creating separate subgroups of keratin filaments in intermediate filament classification (10).

In the present study, developing cortical cells found in anagen follicles of human hair (Fig. 1) were obtained by a procedure using microdissection and subsequently lysed by changing their osmotic environment (11). In this manner we have identified intracellular dispersions containing individual hard α-keratin filaments and organized fibrillar structures (macrofibrils) in electron microscopical preparations. In addition, scattered among macrofibrillar arms, sharply defined, sometimes regularly arranged rectangular structures were observed alternating with darker stained regions. Efforts to evaluate suggestions that these structures were regularly arranged within macrofibrils were hindered by dispersive effects encountered during cell lysis and preparation for electron microscopy. The lightly staining rectangles appeared to lack internal structure at low magnification but when studied in greater detail were found to contain filaments closely packed into longitudinal tactoid-like structures and were termed filament assemblies. The demonstration of filament assemblies in longitudinal sections has eliminated the possibility of their arising as artifacts from the isolation or negative staining procedures. These assemblies provide a new and significant structural element for undertaking direct observations of intramilamentous molecular arrangements in hard α-keratin filaments, and permit an insight into the early stages of filament interactions and their organization in the developing cortical cells of mammalian hairs. Filament assemblies negatively stained with neutral solutions of KPT have demonstrated a repeating axial period in the range 20–22 nm (Fig. 6). A recent model proposed for hard α-keratin filaments devised from x-ray diffraction studies (21) is based on a ring-core substructure (13, 14). Units of coiled molecules are arranged around the outer ring in a helical structure with a 22-nm pitch. In other observations the α-helical domains of IFs (15, 16) show similar packing and pitch lengths. Observations of a 20–22-nm repeating structure on these isolated filament assemblies provides direct evidence to support the x-ray-based model for hard α-keratin filaments and is in agreement with current views (10, 21, 22), which suggests that there is a common structural framework in IFs.

Axial banding extending across the filament assembly is indicative of filaments packing in lateral register. Juxtaposed filaments may be linked via an accumulation of molecular ends that project from their surfaces on the aforementioned
helical ribbon. Neither interfilamentous linkages nor filament assemblies have previously been observed in IFs, but the mechanical behavior in materials that contain hard α-keratin filament arrangements is consistent with the presence of these moieties (23, 24). Recently it has been suggested that the initial stage in the formation of the filament–matrix complex is the assembly of filaments into a lattice via interactions between projections from their surfaces (25). Matrix proteins
FIGURE 5 Unidirectional shadowing with platinum-carbon. A–C show individual hard α-keratin filaments that demonstrate axial repeats at intervals along each filament. D A filament assembly (arrow) contains closely packed filaments that appear as longitudinal striations. Bars, (A–C) 0.01 µm and (D) 0.1 µm. (A–C) × 200,000. (D) × 130,000.

FIGURE 6 (A–C) Filament assemblies viewed after negative staining with neutral solutions of KPT and washing in n-hexane containing cetyl alcohol. Clearly defined axial striations extend across the width of filament assemblies, are regularly spaced, and demonstrate a periodic repeat ranging from 20 to 22 nm. This suggests that hard α-keratin filaments pack in register via the nonhelical molecular ends of component molecules, which may also act as interfilamentous linkages. Bars, 0.1 µm. (A) × 180,000. (B) × 190,000. (C) × 160,000.

FIGURE 7 Two-dimensional polyacrylamide electrophoretic patterns obtained from SCM extract of the whole human follicle (A) and SCM-filament pellet obtained by ultracentrifugation (B). The human follicle contains the hard mammalian keratin protein groups denoted L.S.P. (low sulfur) and H.S.P. (high sulfur) in A. The filament pellet (B) containing 7.0-nm hard keratin filaments and filament assemblies consists of low-sulfur proteins. High-sulfur proteins were not observed in extended exposures to [14C]iodoacetate-labeled extracts of filament pellets. The composition of labeled material of low molecular weight (arrow) present in both extracts (A and B) is unknown.

assemble in the interfilamentous spaces either concurrently or sequentially. Such unique demands on α-keratin filament proteins may account partly for the notable complexity found in these systems.

In the early follicle cells of developing wool (26) and hairs (27) initial keratin deposits are identified as discrete circular areas that form an outer ring within the cell membrane. These deposits appear to contain circular arrays of α-keratin filaments (28). The filament assemblies isolated in the present study originate from the initial filament arrays observed at this level of the follicle. Such structures were found infrequently in earlier studies (11, 12) in which this lower region was not included in the isolation protocol. Their significance was unknown. The only available evidence of filament interaction and filament-associated substances has been from elec-
tron microscopical studies of follicle sections (1, 27-31). These showed that at higher levels of the follicle densely staining inclusions (presumably matrix proteins) become associated with the filamentous arrays. The inflow of this interfilamentous material would mask the presently observed filament interactions. Filament assemblies indicate that the first stages of interfilament attachments may be part of the filament orientation process since they appear concomitantly with cell elongation.

In α-keratin-containing appendages two clearly defined filament packing arrangements have been observed (29), and these are most readily distinguished in the semicircular segments found in the cortex of fine wool fibers (30, 31). In the so-called para-cortical segments, cells contain α-keratin filaments, assuming an orientation parallel with the longitudinal axis of the fiber, and the filaments are arranged in a hexagonal manner. The segment comprising the ortho-cortex contains cells in which microfibrils consist of concentric layers of filaments that appear inclined to the fiber axis when viewed in cross-section. Hence ortho-cell microfibrils have a spiral form.

The isolated filament assemblies discussed here demonstrate longitudinal striations (see Fig. 3) resulting from closely packed α-keratin filaments, or inclined to the longitudinal axis, assuming that the filaments were packed into a cylinder. Where filaments are inclined with the longitudinal axis a spiral or helical arrangement will result, giving rise to a periodic structure. The pitch of this presumed repeating structure was calculated to be ~1,000 nm based on averaged angles of filament inclination and lateral width of filament assemblies, and such a structure would be consistent with the ortho-type whorls observed in cross-sections (26-31).

The filaments and filament assemblies isolated from presumptive cortical cells have been shown to consist entirely of hard mammalian keratin low-sulfur proteins, but this does not exclude the possibility that other proteins such as high-sulfur proteins could become associated with them at a later stage of the keratinization process. The pellet obtained here after ultracentrifugation contains filaments and filament assemblies in unknown proportions, and this fact must be considered in assessing the protein composition of filament assemblies (Fig. 7 B). However, the micrographs presented in Fig. 2 indicate the latter to be a significant component of the pellet. The method of detection for sulfur containing proteins ("[^14]Cjodoacetic acid) provides a sensitive indicator for the presence of S-carboxymethylcysteine. Since high-sulfur proteins of human hair contain three to four times the half-cysteine content (determined as S-carboxymethylcysteine) of low-sulfur proteins (32) they would be detectable if present in filament assemblies even as a small proportion. Extended exposures of electrophoresed extracts were used to check for trace amounts of high-sulfur proteins, but these were not detected. The extracts of follicle control and filament pellet (Fig. 7 A and B) contain labeled material of low molecular weight of unknown composition. The possibility that this material consisted of the high-tyrosine class of hard keratin proteins can be excluded; these proteins do not occur in extracts of human hair (32). If this peptide-like material were associated with the filament assemblies it would represent a new class of low molecular weight proteins not yet identified in mammalian hard keratins.

In summary, tacoid-like filament assemblies composed of hard α-keratin filaments that arise from the initial filament arrays observed in cross-sections of mammalian hair follicles have been isolated. Two types of assemblies are distinguished; the para-like or hexagonally packed array and the ortho-like spiral or whorl type. The demonstration of axial banding extending across these assemblies suggests that filaments are packed into lateral register, possibly via interfilamentous linkages. A regularly spaced 20-22-nm axial banding pattern was observed, which is consistent with the 22-nm periodic structure proposed in models of hard α-keratin filaments and other intermediate filaments. The filament and assembly preparations were shown to contain low-sulfur hard keratin proteins, and no evidence for association of high-sulfur proteins was obtained.

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