terated fluorotyrosyl residues in the presence and absence of nucleotides should reveal more details of the tyrosyl–solvent, tyrosyl–nucleotide interactions.

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Structural Features of the α-Type Filaments of the Inner Root Sheath Cells of the Guinea Pig Hair Follicle†

Peter M. Steinert

ABSTRACT: Since the protein filaments of the mature cells of the inner root sheath of the guinea pig hair follicle are cross-linked by the isopeptide bond ϵ-(γ-glutamyl)lysine, they may only be released from the tissue by brief trypsinization. The filaments are obtained in 60% yield (dry wt), are long tubes 70–80 Å in diameter, and contain citrulline, cross-link and 37% α helix. While this isolation procedure necessarily inflicts some damage, on dissociation two proteins were recovered from the filaments in 60% yield which had molecular weights of 52 000–56 000, contained 45–50% α helix, 28% of the total citrulline but only traces of cross-link. These proteins are probably fragments of the filament subunit(s), but estimates of the numbers or sizes of the intact subunits have not been possible. Further trypsin digestion of isolated filaments released two α-helix-enriched fragments which contained little citrulline and no cross-link. The smaller, particle 2, had a molecular weight of 40 000, an α-helix content of 85%, and dimensions of 170 × 20 Å and accounts for all of the α-helix of the filaments. It consists of a family of molecules each containing three chains of molecular weight 13 000–14 000 aligned side by side which presumably adopt a coiled-coil conformation. This coiled-coil segment or discrete region in the filaments is responsible for the α-type X-ray diffraction pattern given by the filaments. The larger, particle 1, had a molecular weight of about 100 000, contained 71% α helix, had dimensions of 400 × 20 Å, and can also account for all of the α-helix of the filaments. It contains three chains of molecular weight 32 000–35 000 and consists of two segments like particle 2 separated by a region of nonhelix. This particle may represent a major portion of a three-chain unit of the inner root sheath filament. Since the isolated filament proteins and α-helix-enriched particles contain only minor amounts of the citrulline and cross-link of the filaments, it is concluded that the majority of the citrulline and cross-link are located in regions of nonhelix on the filament subunits which are cleaved during the release of the filaments from the inner root sheaths.

The hair follicle is a complex structure in which several distinctly different cell types develop from a common cell population (Fraser et al., 1972). The most prominent are the cell types which comprise the rising column of the hair fiber (fiber cuticle, cortex, and in many coarser hairs the medulla). External to these is a concentric sheath of cells, the inner root sheath. This structure does not emerge from the surface of the skin with the growing hair and is degraded by proteolytic enzymes in the pilary canal (Gemmell & Chapman, 1971). The inner root sheath cells differentiate at an early stage and become filled with a fibrous protein that perhaps derives from (Rogers, 1964a) or admixes with (Parakkal & Matoltsy, 1964) an amorphous protein, trichohyalin. The terminally differentiated inner root sheath cells are packed with the fibrous pro-

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tein and form a hardened, dead, annular column of tissue. The function of the inner root sheath is primarily mechanical; it is thought to constrain the growing hair in the follicle by promoting vertical elongation of the hair cortical cells as the fiber develops (Straile, 1965).

The proteins of the mature inner root sheath have long been known to be unusual; notably, they contain the amino acid citrulline and they are insoluble in the normal solvents for proteins and the denaturing solvents which dissolve hair and the hair follicle (Rogers, 1958, 1959, 1964a,b). The established procedure for the isolation of the proteins was by proteolytic digestion which released the total protein as soluble polypeptides (Rogers, 1962, 1964a,b). More recently, the isopeptide cross-link ϵ-(γ-glutamyl)lysine was found in these peptides (Harding & Rogers, 1971). Since this cross-link is not cleaved by proteolytic enzymes or moderate chemical procedures (Folk & Finlayson, 1977), it adequately explains the insolubility of the proteins. However, on limited exposure of inner root sheaths to a proteolytic enzyme, it was possible to isolate morphologically intact filaments (Steinert et al., 1971) which appeared as long tubes 70–80 Å in diameter and, interestingly, contained citrulline. Presumably, the enzyme cleaved a sufficient number of peptide bonds in the vicinity of the cross-links to release the filaments without completely degrading them. Despite evident damage to the filament protein subunits by this method of isolation, structural studies on the fibrous protein have been possible and are reported in this paper.

Materials and Methods

Isolation of Filaments. Inner root sheaths were isolated by dispersion of the hair follicles of young albino guinea pigs (less than 2 weeks of age) in a buffer of 8 M urea, 10 mM Tris-HCl[1] (pH 7.4) containing 25 mM 2-mercaptoethanol (Steinert et al., 1971). To release the filaments, a suspension of purified sheaths (5 mg/mL in 10 mM Tris-HCl buffer, pH 7.4) was digested with 1 mg/mL trypsin (Sigma Chemical Co., type IX) with stirring. Crystalline trypsin was used here in preference to the impure Difco preparation favored in the earlier study (Steinert et al., 1971) to affect greater reproducibility and yield. Digestion was conducted at 20 °C for 10 min and terminated with a 1.5-fold molar excess of trypsin inhibitor (Sigma, type 1-S). The resulting suspension of inner root sheath cells was washed in buffer three times at 4 °C to remove enzyme, resuspended in the original volume of buffer, homogenized in a Dounce vessel (clearance about 0.07 mm), and centrifuged for 15 min at 10 000*g* to pellet debris. Filaments were retained in the supernatant in a yield of 60–65% (dry wt). Examination is an electron microscope showed very few nonfilamentous particles (Steinert et al., 1971). The tissue debris contained large membranous particles with few attached filaments. Thus, most of the intracellular filaments had apparently been selectively removed. In most experiments, the filaments were harvested at 100 000*g* for 30 min and the clear pellet was resuspended as required by homogenization.

Denaturation of Filaments. Pelleted filaments were dissolved in 8 M urea, 0.1 M Tris-HCl buffer (pH 8.5) containing 25 mM 2-mercaptoethanol (10 mg/mL) with stirring for 1 h at 20 °C, and any sulfhydryl groups present on the protein were converted to their *S*-carboxymethyl derivatives with iodo[2-^{3}H]acetic acid (Amersham-Searle, specific activity reduced to 650 cpm/nmol) (Steinert & Idler, 1975). The protein was equilibrated by dialysis into 0.05 M NaCl, 0.05 M Tris-HCl buffer (pH 7.4) containing 0.1% sodium dodecyl sulfate ($NaDodSO_4$)[1] and chromatographed in the same buffer on a 95 × 2.0 cm column of defined (Kawata & Chase, 1968) Sephadex G-200 at 12 mL/h and collected into 3.0-mL fractions. In some experiments, 6 M guanidine hydrochloride was used for dissociation and chromatography.

Analytical Procedures. For amino acid analysis, proteins were hydrolyzed in 5.7 N hydrochloric acid for 22 h in vacuo and analyzed using lithium citrate buffers on a Beckman 119CL analyzer equipped with a Model 126 data reduction system. To affect separation of citrulline, ornithine, and the cross-link ϵ-(γ-glutamyl)lysine from the other amino acids, the pH of buffer B was lowered by 0.35, and in this system the cross-link eluted at about 124 min. Citrulline values were corrected for the degradation to ornithine that occurs during acid hydrolysis. Tryptophan was estimated colorimetrically (Gaitonde & Dovey, 1970). *S*-Carboxymethylcysteine was determined from the amount of radioactivity in the protein samples. Since the cross-link is hydrolyzed in acid to glutamic acid and lysine, it was released intact by total enzymatic digestion of protein samples (0.5–1.0 mg) with the combination of enzymes used earlier (Harding & Rogers, 1976), and the deproteinized digest was subjected to amino acid analysis. The relative molar amount of cross-link was calculated by relation to the citrulline content, which was also completely released by digestion (Steinert et al., 1969). Similar digests of bovine serum albumin, bovine epidermal α-keratin (Steinert, 1975), or of the enzymes alone, none of which has been reported to contain citrulline or the cross-link, yielded values of less than 0.1 residue of citrulline or cross-link/1000 amino acid residues.

Protein was estimated by the method of Bramhall et al. (1969) using bovine serum albumin as the standard.

The multiphasic discontinuous system with 0.1% $NaDodSO_4$ or 6 M urea was used for polyacrylamide gel electrophoresis (Steinert & Idler, 1975). Molecular weights were determined using appropriate standards by construction of Ferguson plots (Steinert & Idler, 1975). Gels were stained with Xylene brilliant cyanin G in 7.5% trichloroacetic acid. The two prominent proteins of the filaments were separated on 7% T, 3% C preparative $NaDodSO_4$ gels (Steinert & Idler, 1975).

Amino-terminal amino acids were identified and quantitated using [methyl-^{3}H]dansyl chloride (New England Nuclear, specific activity reduced to 470 cpm/pmol) (Woods & Wang, 1966). Amino acids labeled this way were released by acid hydrolysis, mixed with 0.25-nmol amounts of unlabeled dansyl amino acids to facilitate detection, and separated by two-dimensional thin-layer chromatography on polyamide sheets. Spots were cut out, eluted with 1.0 mL of acetone, and mixed with Aquasol (New England Nuclear) for counting. Corrections were made for hydrolytic losses of several acid-labile dansyl amino acids. Carboxyl-terminal amino acids were released with carboxypeptidases A and B and characterized by amino acid analysis.

Isolation of α-Helix-Enriched Fragments. These were prepared by further digestion of isolated filaments. Pellets of filaments were resuspended [5 mg/mL in 0.05 M KCl, 0.05 M sodium tetraborate (pH 9.2)] and digested with trypsin (Sigma, type III) using an enzyme to protein ratio of 1:100 at 20 °C. Aliquots removed at various times were terminated with a 1.5-fold molar excess of trypsin inhibitor and then chromatographed in the borate buffer on a 95 × 2.5 cm column of Sepharose 6B at 30 mL/h and collected into 10-mL fractions. α-Helix-enriched protein was concentrated by precipitation at pH 5 by addition of 0.3 volume of 1.0 M sodium acetate buffer and redissolved in the borate buffer.

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; CD, circular dichroism; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride.

TABLE I: Amino Acid Content of Inner Root Sheath Filaments and Derived Proteins.

amino acid or property	residues/1000 residues: total tryptic pept	whole filaments	protein eluted from Sephadex G-200 at: V_e	filament proteins a	filament proteins b	V_0	V_t
S-CM Cys	6	7	3	2	3	8	15
Asp	93	100	108	112	102	104	66
Thr	29	32	26	30	27	38	60
Ser	58	65	45	42	45	68	98
Glu	222	240	217	213	223	255	258
Pro	35	41	29	28	25	45	62
Gly	74	77	54	55	53	84	113
Ala	61	66	81	86	82	65	27
citrulline[a]	31	39	11	11 (5)	9 (4)	41	130
Val	47	48	66	63	64	46	13
Met	21	20	12	11	10	26	29
Ile	33	34	46	48	50	27	10
Leu	90	98	119	121	124	94	46
Tyr[a]	27	19	24	20 (10)	32 (15)	15	10
Phe	31	17	23	24	25	13	9
Lys	87	46	62	60	64	36	18
His	14	14	13	12	14	15	12
Arg	35	38	50	55	52	34	21
Trp	3	2	2	1	2	2	3
cross-link[a]	2.3	1.8	0.4	0.4 (0.2)	0.2 (0.1)	1.7	2.6
α helix (% ± SD)	14 ± 5	37 ± 3	48 ± 4	45 ± 4	49 ± 3	32 ± 5	<5
total wt of α-helix				25 000	25 000		

[a] The probable number of residues/mole are shown in parentheses.

Electron Microscopy. Specimens were examined after negative staining with 0.7% uranyl acetate on holey ("lacey film") grids (Ladd Research Industries) in a Siemens Elmiskop 1A electron microscope. Statistical data on particle lengths were obtained as before (Steinert, 1978).

Physicochemical Measurements. All studies were done on protein samples dissolved in the borate buffer.

Circular dichroism (CD) was used for the estimation of α-helix contents with a Cary Model 6001 spectropolarimeter using the method of Greenfield & Fasman (1969). When necessary, protein samples were freed of bound $NaDodSO_4$ (Steinert & Idler, 1975).

The intrinsic viscosities of solutions were estimated in an Ostwald-type viscometer (Van Holde, 1971). Outflow times for buffer were 127 s as compared with 147 s for the protein solutions of the lowest concentration used.

Analytical ultracentrifugation was done in a Spinco Model E analytical ultracentrifuge. A close approximation of the $s^\circ_{20,w}$ values was obtained in a single run with solutions of 0.2–1.0 mg/mL using UV-absorption optics with a photoelectric scanner at a speed of 56 000 rpm (Schachman & Edelstein, 1966). Conventional sedimentation equilibrium was studied in 12-mm double-sector cells with 3-mm columns using initial protein concentrations of 0.1–0.5 mg/mL at a speed of 13 000 rpm for 40 h. A weight average molecular weight was calculated by measurement of the optical density along the cells at 0.042-mm intervals and from the known extinction coefficient of the protein and value for $\bar{v}$ derived from the amino acid composition (Schachman, 1957).

A pellet of filaments was used to prepare a fiber suitable for X-ray diffraction as described before (Steinert et al., 1976).

Results

Dissociation of Isolated Filaments. Dissociation of filaments with either 6 M guanidine hydrochloride or 0.1% $NaDodSO_4$ followed by chromatography on Sephadex G-200 resulted in the profile shown in Figure 1. About 60–65% (w/w) of the total protein was recovered in a well-resolved peak when crystalline trypsin was used for the release of the filaments. The crude Difco trypsin gave a similar profile, but the yield of the resolved peak was less and was variable between digests of the same batch of sheaths. This filament protein was used for further studies.

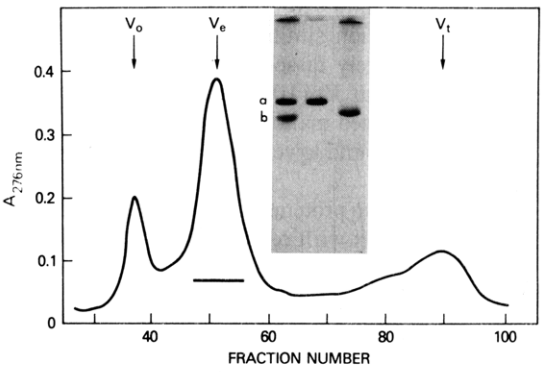

FIGURE 1: Gel filtration of denatured inner root sheath filaments on Sephadex G-200. In this experiment, 50 mg of filaments was dissociated with urea and $NaDodSO_4$ and chromatographed in $NaDodSO_4$ buffer. The bar represents the tubes pooled to obtain the filament protein. Insert: Samples of it were equilibrated in the cathode electrophoresis buffer containing 0.1% $NaDodSO_4$ and then separated on 9% T, 3% C polyacrylamide gels. The two prominent bands were subsequently separated by preparative gel electrophoresis. Gels of protein eluted from the column at V_0 and V_t, respectively, yielded bands of very high molecular weight, most of which did not enter the separation gel, and protein which migrated at the front.

Polyacrylamide gel electrophoresis of it revealed the presence of two broad bands, operationally termed proteins a (larger) and b (Figure 1). Comparison of their mobilities with standard proteins of known size yielded molecular weight estimates of about 56 000 and 52 000, respectively. The two

TABLE II: Yields and α-Helix Contents of the Filament Digestion Products.[a]

time (min)	rel amount and (%) of α-helix content				total α-helix
	V_0	peak 1	peak 2	V_t	
0	1.00 (37)				37
15	0.26 (37)	0.28 (70)	0.08 (80)	0.40 (0)	35
30	0.18 (35)	0.19 (71)	0.20 (81)	0.43 (0)	36
45	0.10 (40)	0.12 (75)	0.31 (85)	0.47 (0)	33
60	0.07 (nd)	0.08 (73)	0.34 (84)	0.51 (0)	34
90	0.05 (nd)	0.05 (nd)	0.30 (81)	0.60 (<5)	24

[a] The data are from the experiment of Figure 3; nd, not determined.

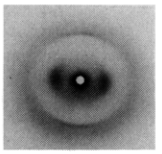

FIGURE 2: X-ray diffraction of inner root sheath filaments.

proteins were separated by preparative polyacrylamide gel electrophoresis and characterized (Table I). Their amino acid compositions were similar, except that protein b contained significantly more tyrosine. In comparison to filaments, the two proteins contained diminished amounts of serine, proline, glycine, and citrulline but elevated amounts of alanine, valine, leucine, lysine, and arginine. Protein that eluted at V_0 from the Sephadex G-200 column (20–25% of the total) possessed an amino acid composition similar to the filaments and thus consists of incompletely dissociated filaments. In contrast, material that eluted at V_t (10–15% of the total) consists of peptides which contained markedly higher amounts of proline, glycine, and citrulline and lower amounts of most other amino acids.

By use of CD the two proteins contained 45–50% α helix and are enriched in α helix with respect to both filaments (37%) and the material eluted from the Sephadex G-200 column at V_0 (32%) and V_t (<5%) (Table I).

The amount of cross-link in the total proteins of the inner root sheath released by tryptic digestion for 6 h was 2.3 residues/1000 amino acid residues (Table I), which is similar to the values of 2.1–2.8 originally reported by Harding & Rogers (1971). Whole filaments contained 1.8 residues/1000 residues, of which on denaturation, most resided in protein eluted at V_0 and V_t. The filament proteins contained 0.2–0.4 residue/1000 residues or 0.1–0.2 residue/mol.

Both filament proteins possessed a total of 1.0–1.2 mol/mol of free amino-terminal amino acids, of which glutamic acid (or glutamine), citrulline, valine, and leucine predominated. Carboxyl-terminal analyses indicated the presence of only lysine and arginine.

Ultrastructural Features of the Filaments. Early X-ray diffraction studies of intact inner root sheaths showed a vague α-type pattern (Rogers, 1959, 1964b). Exposure of a fiber prepared from a pellet of filaments to X radiation gave a typical α-type pattern (Figure 2) which displayed sharp meridional reflections at 5.17 Å and equatorial reflections at 9.8 Å. This establishes the origin of the α-type pattern in the filaments of the inner root sheath. Thus, like other α-type proteins of the k-m-e-f class, the α helix of the subunits of the filaments is arranged in a coiled-coil conformation (Crick, 1952; Pauling & Corey, 1953). These α-helical regions have been demonstrated to be more resistant to mild proteolytic attack than the

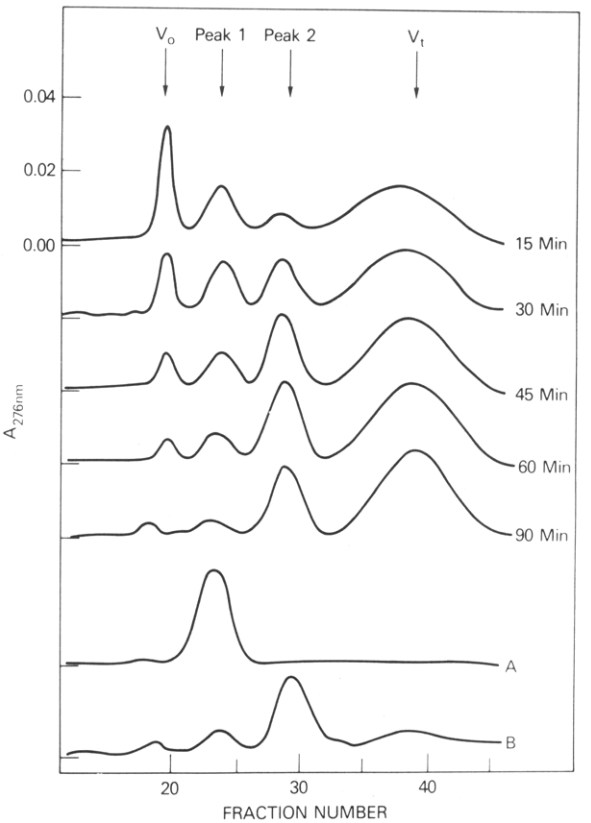

FIGURE 3: Gel filtration of the tryptic digestion products of filaments on Sepharose 6B. About 50 mg of filaments was digested, and terminated aliquots were chromatographed. Two resolved peaks of protein, termed peaks 1 (particle 1) and 2 (particle 2), were recovered by precipitation at pH 5 for further study. In one experiment, of 4 mg of particle 1 at t = 15 min, 2 mg was rechromatographed (trace A) and the remainder was digested for a further 45 min and then rechromatographed (trace B).

neighboring regions of nonhelix in such α-type proteins as myosin (Lowey et al., 1969), the wool "low-sulfur" proteins (Crewther et al., 1971; Lotay & Speakman, 1977), and bovine epidermal keratin filaments (Steinert, 1978). In each of these cases, α-helix-enriched fractions were obtained which provided information on the ultrastructure of the fibrous protein.

Digestion of Filaments with Trypsin. Isolated filaments were digested with trypsin, and at various times aliquots were withdrawn and chromatographed on Sepharose 6B (Figure 3). The yields and α-helix contents of the peaks obtained are shown in Table II. In addition to protein eluted at V_0 and V_t, two resolved peaks which had enriched α-helix contents were recovered. With increasing times (t) of digestion, protein eluted at V_0 and in peak 1 diminished, while that eluted in peak 2 and at V_t increased, suggestive of a precursor–product relationship.

TABLE III: Amino Acid Content of Particles 1 and 2 and the Nonhelical Protein of Particle 1.[a]

amino acid or property	residues/1000 residues		
	particle 1	particle 2	protein eluted at V_t
S-CM-Cys	1		1
Asp	104	109	83
Thr	18	14	38
Ser	33	29	69
Glu	232	243	201
Pro	11	7	47
Gly	31	29	66
Ala	90	94	61
citrulline[b]	3 (2.8)	0.2 (< 0.1)	14
Val	75	78	47
Met	7	6	13
Ile	58	59	47
Leu	131	135	103
Tyr	32	36	24
Phe	29	30	28
Lys[b]	68 (61)	71 (26)	53
His	9	8	14
Arg[b]	64 (58)	63 (22)	59
α-helix-favor.	632	659	495
α-helix-inhib	75	65	182
cross-link			0.1
app mol wt	100 000	40 000	
α-helix (% ± SD)	71 ± 3	85 ± 4	
total wt of α-helix	71 000	34 000	

[a] In the experiment of Figure 3, trace B, the particle 1 before digestion and the particle 2 and material eluted at V_t after digestion were analyzed. [b] Probable numbers of residues per mole are shown in parentheses.

To test this possibility, the protein of peak 1 at t = 15 min was isolated, digested for 45 min with trypsin, and rechromatographed (Figure 3, trace B). Most of the protein and more than 90% of the α helix was recovered in peak 2. To confirm that the highly α-helical protein of peak 2 arose from that of peak 1 by trypsin digestion only, a control sample before digestion was rechromatographed and it eluted in the original tubes (Figure 3, trace A). At all times of digestion prior to about 60 min, 80–95% of the α helix of the filaments could be accounted for (Table II).

Hereafter, the protein of peaks 1 and 2 will, respectively, be termed particles 1 and 2.

Properties of the α-Helix-Enriched Particles. The intrinsic viscosities of particles 1 and 2 isolated at 15 and 60 min of digestion were 12.6 and 7.5, respectively. When considered as prolate ellipsoids of revolution, their axial ratios were 12.0 and 7.1 assuming 30% hydration and 13.0 and 7.9 assuming zero hydration (Mehl, 1940). Using the calculations of Yang (1961), these values correspond to lengths of approximately 400 and 170 Å, respectively.

By electron microscopy, both particles appeared as solid rods about 20 Å in diameter, but particle 1 was about (mean ± SD) 375 ± 75 Å long and particle 2 was 175 ± 35 Å long (Figure 4).

In conventional sedimentation equilibrium experiments, particle 1 behaved as a relatively homogeneous particle of molecular weight about 100 000 between concentrations of 0.2–1.2 mg/mL. Plots of ln c vs. r^2 were linear over about 60% of the cell at each of three initial protein concentrations. In sedimentation velocity experiments, particle 1 sedimented as a single slightly asymmetrical component with an approximate $s^\circ_{20,w}$ value of 4.5. Particle 2 displayed a greater degree of homogeneity, yielding an apparent molecular weight of about

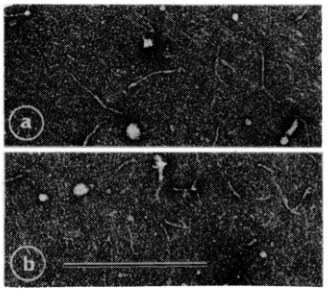

FIGURE 4: Electron microscopy of particles 1 and 2. Samples of (a) particle 1 at t = 15 min and (b) particle 2 at t = 60 min were used. Magnification, ×190 000; the bar is 0.1 μm.

40 000 and sedimented as a single component with an $s^\circ_{20,w}$ value of 3.0.

The amino acid compositions of the two particles are shown in Table III. In comparison to the two filament proteins and filaments, both particles were enriched with respect to the "α-helix-favoring" amino acids, aspartic acid, glutamic acid, alanine, valine, and leucine, and significantly diminished in the "α-helix-inhibiting" amino acids, serine, proline, and glycine (Robson & Pain, 1971). Neither particle contained the cross-link, and only particle 1 contained about three residues/mol of citrulline. In comparison, the amino acid composition of the protein eluted at V_t from the Sepharose 6B column upon the tryptic conversion of particle 1 to particle 2 (Figure 3, trace B) was depleted in the α-helix-favoring amino acids and enriched in the α-helix-inhibiting amino acids and contained all of the citrulline of particle 1 (Table III).

On reaction with [^{3}H]dansyl chloride, particle 2 isolated at t = 60 min contained only 0.9–1.0 mol/mol of glutamic acid

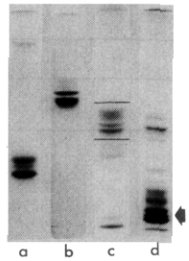

FIGURE 5: Polyacrylamide gel electrophoresis of peptides derived from the α-helix-enriched particles on denaturation. Gel a, particle 2 at t = 60 min was denatured with $NaDodSO_4$ and run on a 12% T, 3% C gel with $NaDodSO_4$; gel b, same sample but denatured with 10 M urea and run on a 9% T, 3% C gel containing 6 M urea; gels c and d, particle 1 at t = 15 and 60 min, respectively, denatured with $NaDodSO_4$ and run on 10% T, 3% C gels with $NaDodSO_4$. The bars on gel c delineate the bands eluted for further study. The arrow on gel d indicates the position of migration of the particle 2 peptides.

(or glutamine), glycine, and serine as the amino-terminal amino acids. Particle 1 isolated at t = 15 min contained a total of 3.3 mol/mol of free amino-terminal amino acids of which (in mol/mol) glutamic acid (0.9), glycine (0.8), and serine (0.7) predominated.

Dissociation of particle 2 with $NaDodSO_4$ followed by polyacrylamide gel electrophoresis revealed three peptide bands which in comparison to the mobilities of standard proteins of known size had molecular weights of 13 000, 13 400, and 14 000 (Figure 5, gel a). When electrophoresed on a urea gel (Figure 5, gel b), several more peptide bands were evident. In particle 1 isolated at t = 15 min, more than 75% of the total peptide material consisted of several bands of molecular weight 32 000–35 000, and the remainder had a molecular weight of 12 000–25 000 (Figure 5, gel c). These larger peptides were eluted, freed of bound $NaDodSO_4$, and characterized. Their amino acid composition was similar to that of particle 1; they contained one residue/average mole of citrulline and did not contain the cross-link. Their α-helix content of 70 ± 4% corresponds to an average of 22–24 000 daltons of α-helix per peptide chain. In particle 1 recovered at t = 60 min, most of the peptides had a molecular weight less than 25 000, and the peptide bands corresponding to those of particle 2 are prominent (Figure 5, gel d).

Discussion

Possible Subunit Structure. The filaments of the mature inner root sheath cells of the hair follicle are cross-linked by the isopeptide bond ε-(γ-glutamyl)lysine (Harding & Rogers, 1971) and it is necessary to cleave this cross-link before they can be released. Since there is no procedure for specifically cleaving it (Folk & Finlayson, 1977), the only known way of releasing the filaments is by brief exposure of sheaths to a proteolytic enzyme. Not surprisingly, the proteins obtained on dissociation of the filaments were partially degraded. Even so, two protein species of molecular weight 52 000 and 56 000 were recovered, which represented 60% of the total mass of the filaments. It seems likely that these are major fragments of the intact filament subunit(s). The limitations imposed by the method of recovery of the filaments preclude an estimate of the number or size of the intact subunits; nor can the possibility be excluded that the two proteins identified are derived from a single subunit.

Structure of the α-Helix-Enriched Fragments. On further digestion of isolated filaments with trypsin, two α-helix-enriched fragments were obtained which provide information on the ultrastructure of the filaments. The smaller, eluted in peak 2 from the Sepharose 6B column (particle 2), had an α-helix content of about 85% and a molecular weight of 40 000. It contained several peptides of molecular weight 13 000–14 000 (Figure 5) which is one-third of its molecular weight. Therefore, intact particle 2 consists of a family of similar molecules each containing three peptide chains. Particle 2 contained about 34 000 daltons of α helix (Table III) or about 11 000–12 000 daltons per peptide chain. From the mean residue weight of 111, there are about 120 amino acids in each peptide of which 100–110 are in the α-helical conformation. As each amino acid in an α helix has a linear displacement of 1.5 Å, the total length of each peptide is near 120 × 1.5 = 180 Å. Clearly, this is about the same length as intact particle 2, and so the three peptide chains are aligned side by side. Presumably, these adopt a coiled-coil conformation which is responsible for the characteristic α-type X-ray diffraction pattern given by the filaments (Figure 2). Such a conformation would be expected to render the 48 lysine and arginine residues of particle 2 (Table III) inaccessible to tryptic digestion. Since by 60 min of digestion more than 75% of the α helix of the filaments was present as particle 2 (Table II), it is likely that all of the α helix of the filaments is present as these discrete three-chained coiled-coil segments.

Interestingly, three-chained coiled-coil α-helical particles of similar dimensions and properties have been isolated by limited proteolysis of several α-keratin proteins: wool "low-sulfur" proteins (Crewther & Dowling, 1971; Lotay & Speakman, 1977), epidermal prekeratin (Skerrow et al., 1973), and bovine epidermal keratin filaments (Steinert, 1978).

The larger α-helix-enriched fragment, particle 1, degraded on further digestion to particle 2 with retention of all α helix (Figure 3). From its average molecular weight of 100 000 and α-helix content of 71%, it contains 71 000 daltons or twice as much α helix as particle 2 (Table III). On denaturation with $NaDodSO_4$ it initially yielded peptides of one-third the molecular weight (between 32 000 and 35 000), which contained an average of 22 000—24 000 daltons of α-helix or twice as much as the particle 2 peptide chains (Figure 5). Therefore, the most likely structure of particle 1 is that it consists of three chains and contains two segments like particle 2 or about 80 000 molecular weight and an additional 20 000 daltons of mass, which is nonhelix on the basis of amino acid composition data (Table III). One of the α-helical segments is located at the amino-terminal end, since both particles 1 and 2 have common amino termini. The second may be located toward the carboxyl-terminal end by analogy with the structure of a similar particle isolated from bovine epidermal keratin filaments (Steinert, 1978); that is, the 20 000 daltons of nonhelix separates the two α-helical regions. The smaller peptides seen on denaturation of particle 1 especially at the longer times of digestion (Figure 5) probably arise through tryptic cleavage in the central nonhelical region. Each large particle 1 peptide may thus be viewed as two α-helical regions separated by a region of nonhelix.

Possible Ultrastructure of the Filaments. Within the limits of the CD measurements employed, the large particle 1 peptide chains contain the same amount of α helix as the isolated filament proteins (Table I). That is, the filament proteins contain the particle 1 peptides within their sequence and as much as 20 000 daltons of the remaining mass is nonhelix located on one or perhaps both ends. Accordingly, the release of the three-chain particle 1 during digestion of filaments suggests that it derived from a section of the filaments corresponding in size to three adjacent filament proteins or larger (Figure 6). Indeed, it is possible that the intact filament consists of a unit structure of three subunits as in epidermal keratin filaments

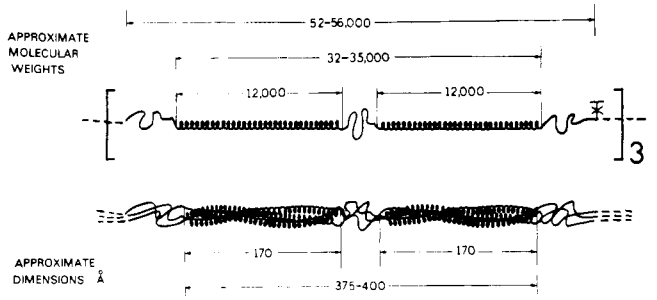

FIGURE 6: Model for the known details of the ultrastructure of the inner root sheath filament. Salient features are: (1) The intact filament subunits are larger than the isolated filament proteins, which have molecular weights of 52 000–56 000. (2) At least that portion including the isolated filament proteins has a three-chain structure. (3) The two α-helical regions of adjacent aligned chains form two discrete three-chain coiled-coil α-helical segments each corresponding to particle 2 and the region including both segments corresponds to particle 1. (4) The majority of the citrulline and all of the cross-link (marked by X) are located in regions of nonhelix distal from the α-helical segments and are concentrated near and beyond the site of cleavage during release of the filaments from the inner root sheaths (denoted by broken lines).

(Steinert, 1978) or a simple multiple thereof as in fibrinogen (Doolittle, 1973). Because the filament proteins are cleavage products of larger protein chains of indeterminate size, it is not known whether such a hypothetical three-chain molecule contains only two regions of coiled-coil α-helix identified here and as in epidermal keratin filaments (Steinert, 1978), or three as in wool (Crewther & Dowling, 1971; Dobb et al., 1973), or more. Unambiguous resolution of this and related ultrastructural questions of the inner root sheath filament may have to await the development of a technique for the specific cleavage of the cross-link. Known details of structure which provide a focus for further studies are summarized in Figure 6.

A prominent feature of the α-helix-enriched particles is the absence of cross-link. The traces of it on the filament proteins (Table I) are thus located in the nonhelical regions. The brief digestion procedure required for the release of the filaments cleaves the protein chains in the more accessible nonhelical regions rather than near the highly ordered coiled-coil α-helical segments. A second and related feature is the diminished content of citrulline. One residue of citrulline per peptide of particle 1 is located in the nonhelical region (Table III). That is, the other three to four residues of citrulline/mol of the filament proteins (Table I) are also located in the nonhelical regions. However, the filaments contain much higher amounts of both citrulline and the cross-link (Table I). As virtually all of the α helix of the filaments can be accounted for in particles 1 and 2 (Table II), more than 70% of the citrulline and 80% of the cross-link must be present in additional nonhelical regions that are cleaved to permit release of the filaments. Fragments of this which were not lost during isolation of the filaments would have eluted at V_t from the Sephadex G-200 column, and, indeed, this material had a high content of α-helix-inhibiting amino acids, citrulline, and cross-link (Table I).

The origin of citrulline in the proteins of the inner root sheath (and hair medulla and related structures) has been ascribed to the desimidation of peptide-bound arginine (Rogers, 1963, 1964a,b; Rogers et al., 1977). Also, transglutaminases which might affect the formation of cross-links between acceptor lysine and glutamine residues have been identified in hair follicles (Chung & Folk, 1972; Harding & Rogers, 1972; Buxman & Wuepper, 1976, 1978). The localization of citrulline and crosslink in the nonhelical regions of the inner root sheath filaments is consistent with the accessibility that would be required by the enzymes responsible for their formation.

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Partial Purification and Characterization of Ribonuclease III Like Enzyme Activity from Cultured Mouse Embryo Cells†

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ABSTRACT: A ribonuclease with properties analogous to *Escherichia coli* RNase III has been partially purified from the cytoplasm of high-passage Swiss mouse embryo (HPME) cells. From 60 to 70% of the cytoplasmic enzyme activity (monitored using [^{3}H]poly(rA)·poly(rU) as substrate) was associated with the ribosomal and microsomal fractions and about 10% of the microsome-associated and 52% of ribosome-associated enzyme activity could be solubilized by 0.4 M KCl. The solubilized enzyme was further purified by ammonium sulfate fractionation and DEAE-cellulose (DE52) column chromatography. The DE52 enzyme degraded single-stranded ribopolynucleotides to an extent of 10-20% under the conditions that resulted in 80-100% solubilization of [^{3}H]poly(rA)·poly(rU). The cleavage of poly(rA)·poly(rU) was maximum at 25-50 mM K^+ and 2-5 mM Mg^{2+} ion concentrations and was inhibited by EDTA or *Penicillium chrysogenum* double-stranded (ds) RNA. The DE52 enzyme fraction showed contaminating hybrid nuclease activity but was devoid of DNase activity. Consistent with our previous observation with a microsome-associated nuclease from the same cell line, the partially purified enzyme endonucleolytically cleaved the polycistronic murine leukemia virus (MLV) 35S RNA. In addition, this enzyme preparation was capable of cleaving 45S ribosomal precursor RNA and the cleavage products had electrophoretic mobilities similar to those of mature ribosomal RNAs and their precursor intermediates.

Several different nucleases appear to be involved in the processing of cellular and viral RNAs (Perry, 1976; Robertson & Dickson, 1975). Ribosomal RNAs, mRNAs, and tRNAs are transcribed in the form of high-molecular-weight precursors that are later cleaved by specific nucleases to mature-size products. RNase III, an enzyme specific for double-stranded regions of RNA (Robertson et al., 1968), has been implicated in the processing of *Escherichia coli* rRNA precursors and bacteriophage T7 mRNA transcripts (Dunn & Studier, 1973). Another enzyme, RNase P, was shown to process tRNA precursors in *E. coli* (Robertson et al., 1972). Since RNA processing enzymes might be expected in eukaryotic cells due to the fact that they also contain RNA precursors which give rise to smaller mature RNAs, recent investigations have attempted to detect and study the role of these enzymes in the maturation of cellular RNAs (Rech et al., 1976; Ohtsuki et al., 1977; Hall & Crouch, 1977).

Our interest in RNA processing enzymes had an additional objective aimed at understanding the role of nucleases in the processing of polycistronic animal viral mRNAs. There appears to be precedence for a possible involvement of cytoplasmic nucleases in the processing of SV40 specific 19S mRNA to 16S mRNA in enucleated cells (Aloni et al., 1975). Therefore we initiated a search for eukaryotic nucleases that may be involved in the processing of viral RNAs (Shanmugam, 1976). These early studies indicated the presence of an RNase III like enzyme activity in the microsomal fraction of uninfected and oncornavirus infected cells which may be involved in the generation of the 20S virus-specific RNA species present in the microsomes (Shanmugam et al., 1974). Leis et al. (1978) recently identified a similar enzyme activity in duck embryo microsomes which cleaved Rous sarcoma virus 34S RNA. In both studies (Shanmugam, 1976; Leis et al., 1978) the microsome-associated particulate enzyme was used to cleave the oncornavirus RNAs. In studies described here, this ds RNase activity has been solubilized and partially purified from the cytoplasm of cultured mouse embryo cells. The partially purified enzyme has several properties (such as ribosomal association, ionic requirements, and preference for dsRNA) resembling *E. coli* RNase III and endonucleolytically cleaves nucleolar 45S ribosomal precursor RNA and polycistronic MLV RNA.

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