BOVINE EPIDERMAL KERATIN FILAMENT ASSEMBLY *IN VITRO*

Peter M. Steinert and Marisa I. Gullino

Dermatology Branch
National Cancer Institute
Bethesda Maryland 20014

Received February 4,1976

SUMMARY: The polypeptides of bovine epidermal α-keratin, which comprise the subunits of the keratin filaments *in situ*, reassemble into filaments spontaneously *in vitro* in dilute salt solution and in the absence of dissociating reagents and additional cofactors. The polypeptide composition of these filaments, and their structure, based on electron microscopy and X-ray diffraction, are the same as the *in situ* keratin filaments.

The major differentiation product of mammalian epidermis is an α-fibrous protein (α-keratin) which appears intracellularly in the form of tonofilaments (keratin filaments) (1). This component ultimately forms the bulk of the outer, dead layer of the epidermis, the stratum corneum. When seen in thin section in an electron microscope, the keratin filaments are 70-80 Å in diameter but of indeterminate length since they are not well-aligned in the plane of the section (2,3). The upper limit of their length may be the size of the cell (10-30 μm). Treatment of either the living epidermal cells or the stratum corneum with an 8 M urea buffer at neutral or alkaline pH dissociates the keratin filaments, releasing their constituent polypeptide chain subunits (4,5). These polypeptides can assemble *in vitro* into filamentous particles (5). In this report, we extend this finding to show that the chemistry and structure of the filaments is the same as the *in situ* keratin filaments.

EXPERIMENTAL

Tissue containing the living cells of bovine epidermis from the posterior region of the hoof was extracted in a buffer of 8 M urea, 0.05 M Tris-HCl (pH 9.0) and 0.025 M 2-mercaptoethanol (25 ml/g wet wt.) for 3 hr at 37°. The solution was centrifuged at 30,000 x g for 30 min to remove cellular debris and then at 250,000 x g for 2 hr to remove smaller particles. Filaments were formed from the α-keratin polypeptides in this solution.

For electron microscopy, filaments were negatively-stained with 0.7 % uranyl acetate on holey grids and examined in a Siemens 1A microscope.

Polyacrylamide gels (9 % T, 3 % C) with 0.1 % sodium dodecyl sulfate (SDS) were used (6). Samples were equilibrated in the cathode buffer containing SDS by dialysis for 18 hr, heated at 95° for 2 min to effect complete binding of SDS (6), electrophoresed for 3 hr at 2 mA/gel and stained with Fast Green.

For X-ray diffraction, pellets of filaments were drawn onto a cross-wire support and allowed to dry at 5° into a fiber 20-30 μm in diameter. The fiber was exposed to Cu-K_α radiation for 48 hr. For calibration purposes, the same fiber was dusted with NaCl crystals and re-exposed.

RESULTS AND DISCUSSION

Filaments were produced *in vitro* by dialysis of urea extracts against a dilute salt solution ($I < 0.010$ mol/l) such as 5 mM Tris-HCl at pH 8.0. At an ionic strength above 0.01 mol/l, the polypeptides precipitated from solution and filaments did not form. The filaments (Figure 1) are of uniform diameter of 70-80 Å but their length was dependent on the presence of a reducing agent (2-mercaptoethanol) in the dialysis buffer. In its absence (Figure 1a), the filaments were 0.2-1.0 μm in length, but in the presence of 0.025 M 2-mercaptoethanol, the filaments were much longer (Figure 1b). Measurement of numerous particles in adjacent overlapping photographs indicated an average ± s.d. length of 15.7 ± 3.5 μm. Short filaments in suspension that had been formed in the absence of 2-mercaptoethanol could be converted within 1 hr to very long filaments by addition to the solution of 0.1 M 2-mercaptoethanol (final concentration). The dimensions of the filaments assembled *in vitro* appear to be within the range of the keratin filaments seen *in situ*. However, attainment *in vitro* of the *in situ* lengths appears to be dependent on maintainence of the thiol groups on the polypeptides in the fully-reduced form. A previous report (5) showed that the thiol groups of the polypeptides become oxidized to disulfide bonds *in vitro* and it seems reasonable to postulate that random formation of disulfide bonds inhibits filament assembly. This is consistent with the *in situ* situation; the keratin filaments are initially deposited and are maintained in their thiol forms for some time before finally becoming oxidized to disulfide bonds during terminal stages of differentiation.

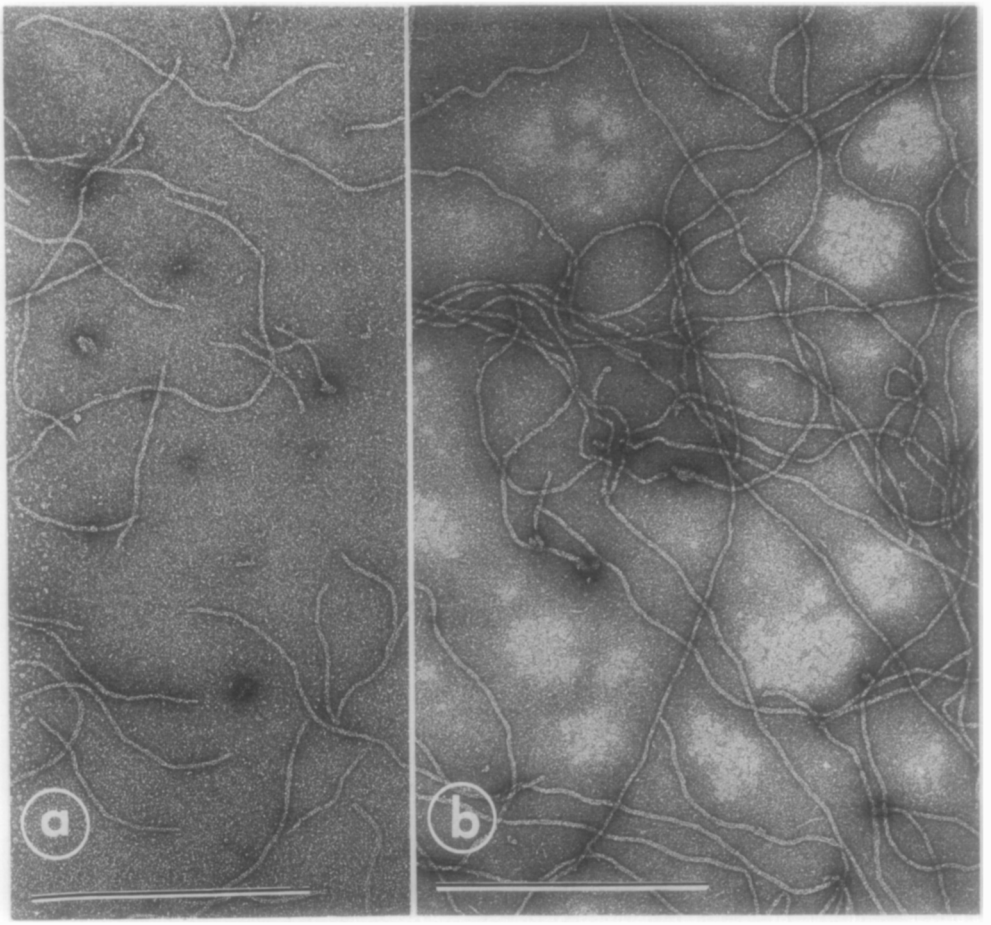

Figure 1. Electron microscopy of assembled filaments. A solution of polypeptides was diluted to about 2 mg/ml with the urea buffer and dialzed against 1000 vol. of 5 mM Tris-HCl (pH 8.0) in the absence a and presence b of 0.025 M 2-mercaptoethanol for 24 hr at 23°. Samples of the solutions which by this time contained the filaments were examined after negative staining. Calibration bar is 0.5 μm.

When filaments in salt solution with or without 2-mercaptoethanol were sedimented from suspension by ultracentrifugation at 250,000 x g for 1 hr, more than 90 % of the total protein in solution was pelleted. This yield of polypeptides in filaments was independent of the polypeptide concentration between 0.1-10.0 mg/ml and of the temperature between 1-37°.

Above 10 mg/ml, the filaments formed a translucent gel which was difficult to pellet. However, when the dialysis buffer contained 8 M urea, 6 M guanidine hydrochloride or 0.1 % SDS, less than 1 % of the total protein in solution could be pelleted. Furthermore, addition of any of these reagents to suspensions of preformed filaments resulted in the rapid dissociation of the filaments within 1 hr, as no material could then be pelleted. Therefore, it is apparent that the assembly and structure of the filaments are mediated by secondary (non-covalent) bonds since the reagents classically interfere with such types of interactions.

Filaments that had been pelleted by ultracentrifugation could also be readily dissociated (depolymerized) by the urea buffer, and on dialysis of the urea solution into 5 mM Tris-HCl (pH 8.0), filaments were reformed (repolymerized) that appeared the same in the electron microscope. This cyclic process of polypeptide polymerization, depolymerization, and then repolymerization could be carried out several times on the same sample of polypeptides.

On polyacrylamide gels with SDS, the α-keratin polypeptides extracted from the epidermis separate as six bands which have molecular weights within the range of 47-58,000 (5,6). The assembled filaments also contained all six of the bands of polypeptides, and moreover, the relative amounts of each band were the same (Figure 2). These band patterns were unchanged after several cycles of depolymerization and repolymerization (Figure 2). Thus the assembled filaments have the same polypeptide composition as the in situ keratin filaments. X-ray diffraction analysis of the assembled filaments (Figure 3) demonstrates an equitorial reflection at 9.8 Å and a sharp meridional reflection at 5.15 Å. This pattern is attributed to reflections from a coiled-coil arrangement of two or three α-helices and is considered characterisitic of epidermal α-keratin (7-9).

In additional experiments, 1-2 mM $MgCl_2$, 1-2 mM $CaCl_2$, 1 M sucrose, 10-40 % glycerol, 0.1-1.0 mM colchicine, 0.1-1.0 mM ATP or GTP were added

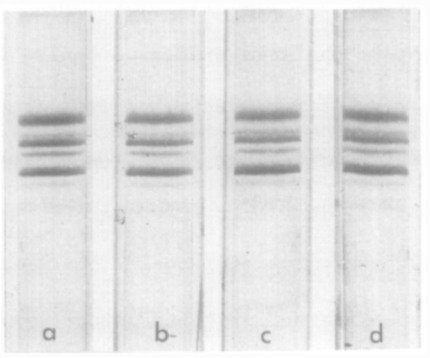

Figure 2. SDS polyacrylamide gels of assembled filaments. a α-Keratin polypeptides extracted from the epidermis. b Polypeptides of assembled filaments. Filaments prepared as in Figure 1b were pelleted (250,000 x g, 1 hr) and dissolved (depolymerized) in the urea buffer. c Polypeptides of repolymerized filaments. As in b but the solution of depolymerized filaments in urea was centrifuged (250,000 x g, 2 hr) and the supernatant still containing more than 98 % of the total protein was dialyzed as in Figure 1b. The repolymerized filaments which formed were pelleted (250,000 x g, 1 hr) and depolymerized in urea again. d Polypeptides of filaments repolymerized a second time. As in c except that this process was repeated another time.

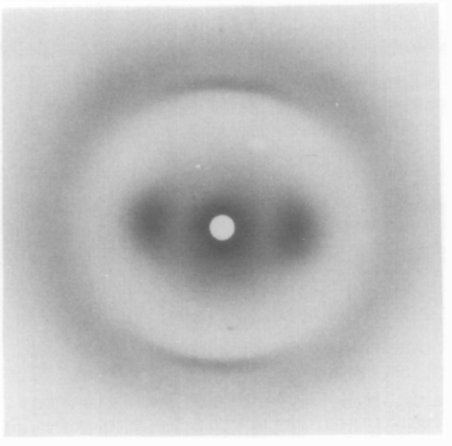

Figure 3. X-ray diffraction of assembled filaments. The fiber used to obtain this pattern was made from filaments that had been assembled as in Figure 1b.

to either the dialysis buffer or to suspensions of preformed filaments. None of these reagents was required for filament assembly, or modified the structure of preformed filaments, using as criteria, length, width and yields

of polypeptides in filaments. In contrast, some of these reagents are either required for the *in vitro* assembly of or modify the structure of microtubules (10,11) or neurofilaments (12). This distinguishes the epidermal filaments from these types of filamentous particles.

The evidence favors the view that the filaments assembled *in vitro* are the same as the *in situ* keratin filaments. One other report has described the assembly of epidermal proteins into filaments (13) but few experimental details were provided. Other workers have also alluded to this phenomenon (14-16). The present study constitutes the first detailed report of the self-assembly of epidermal α-keratin into filaments *in vitro*. As the individual polypeptide subunits of epidermal α-keratin have now been separated (6), this self-assembly process will provide a useful method for analyzing the ultrastructure of the epidermal keratin filament.

ACKNOWLEDGEMENTS

We thank Dr. Steven Zimmerman for the X-ray diffraction study, Dr. Gary Peck for thoughtful advice and Mr. William Idler for skilled technical assistance.

REFERENCES

1. Fraser, R.D.B., MacRae, T.M. and Rogers, G.E. (1972) Keratins Their Composition, Structure and Biosynthesis, Charles C. Thomas, Springfield, Illinois.
2. Brody, I. (1960) J. Ultrastruct. Res., 4, 264-297.
3. Roth, S.I. and Clark, W.H. (1964) in The Epidermis, Montagna, W.W. and Lobitz, W.C., eds., pp. 303-337, Academic Press, New York.
4. Baden, H.P., Goldsmith, L.A. and Fleming, B. (1973) Biochim. Biophys. Acta, 317, 303-311.
5. Steinert, P.M. (1975) Biochem. J., 149, 39-48.
6. Steinert, P.M. and Idler, W.W. (1975) Biochem. J., 151, 603-614.
7. Crick, F.H.C. (1952) Nature, 170, 882-883.
8. Pauling, L. and Corey, R.B. (1953) Nature, 171, 59-61.
9. Fraser, R.D.B., MacRae, T.M., Millward, G.R., Parry, D.A.D., Suzuki, E. and Tulloch, P.A. (1971) Applied Polymer Symp. No.18, pp. 65-83, Wiley, New York.
10. Weisenberg, R.C. (1972) Science, 177, 1104-1105.
11. Shelanski, M.L., Gastin, F. and Cantor, C.R. (1973) Proc. Nat. Acad. Sci., 70, 765-768.

12. Gilbert, D.S., Newby, B.S. and Anderson, B.H. (1975) Nature, 256, 586-589.
13. Matoltsy, A.G. (1965) in Biology of the Skin and Hair Growth, Lyne, A.G. and Short, B.F., eds., pp. 291-305, Angus and Robertson, Sydney.
14. Baden, H.P., Gifford, A.M. and Goldsmith, L.A. (1971) J. Invest. Dermatol., 56, 446-449.
15. Tezuka, T. and Freedberg, I.M. (1972) Biochim. Biophys. Acta, 263, 382-396.
16. Ugel, A.R. and Idler, W.W. (1972) J. Cell Biol., 52, 453-464.