DIVALENT CATION STIMULATION OF IN VITRO FIBRE ASSEMBLY FROM EPIDERMAL KERATIN PROTEIN

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SUMMARY

Keratin was extracted from purified cornified cells of newborn rats in Tris-HCl-buffered 8 M urea containing β-mercaptoethanol. Microfilaments were assembled in vitro by reducing the ionic strength of buffer and the urea concentration.

One millimolar concentration of KCl and NaCl did not affect filament formation, but the same concentration of divalent cations greatly altered this process. CaCl₂ and MgCl₂ induced gelation of keratin by formation of bundles of birefringent macrofilaments. ZnCl₂, CuSO₄ and HgCl₂ formed greater numbers of macrofilaments and the protein aggregated.

INTRODUCTION

Electron microscopy demonstrates the existence of numerous fibres in epidermal cells. In the fully keratinized cornified cells which form the outer cell layers, the majority of filaments are dispersed throughout the cytoplasm and are individually recognizable, either exhibiting a 'keratin pattern' or unassociated with interfibrillar material (Breathnach, 1975; Brody, 1977). It is generally agreed that these keratin filaments are initially formed in basal and spinous cells. The precursor filaments are usually called tonofilaments and are often found as bundles in the cytoplasm. In addition, groups of tonofilaments attach at the plasma membrane and form the desmosome and hemidesmosome complex (Staehelin, 1974; Brysk, Gray & Bernstein, 1977). These bundles persist into the granular cells, but they disperse to become keratin filaments of cornified cells. The biological role of these epidermal fibres is considered to be stabilization of cell shape to form protective layers on the body's surface.

Proteins of the epidermal fibres have been isolated and purified from human (Baden & Bonar, 1968; Bauer, 1972), cow (Rudall, 1952; Matoltsy, 1964), mouse (Carruthers, 1974), and newborn rat (Shimizu, Fukuyama & Epstein, 1974; Huang, Stern, Clagett & Chi, 1975). Proteins of keratin fibres and of tonofilbrils are reported as indistinguishable by some investigators (Matoltsy, 1975; Steinert, 1975) and variable by others (Lee, Fleming, Waitkus & Baden, 1975). Sodium dodecylsulphate

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(SDS)-polyacrylamide gel electrophoresis has demonstrated that the molecular size of polypeptides which constitute epidermal fibres show distinct interspecies differences, although all of them fall in the range of 45,000 to 70,000 Daltons.

Recently, Steinert (1975) and Steinert, Idler & Zimmerman (1976) isolated polypeptides from bovine epidermis and polymerized them into 8 nm wide and 0.1 to 40 μm long fibres morphologically resembling keratin filaments. The polymerization was not influenced by the presence of CaCl₂, NaCl, KCl or MgCl₂ (Steinert et al. 1976). In the present paper we report that proteins isolated from epidermal keratin fibres of newborn rat having different polypeptide composition from bovine fibres also assemble in vitro into fibres and that this assembly is markedly altered by addition of a variety of salts.

MATERIALS AND METHODS

Isolation of keratin

All procedures were done at 4 °C. Skin was removed from 1-2-day-old Sprague-Dawley rats and immersed in 0.24 M NH₄Cl (pH 9.5) for 30 min. The epidermis was separated from the dermis and washed with gentle stirring stepwise in 20 mM Tris-HCl, 0.25 M sucrose (pH 8.0), and then 25 mM Tris-HCl, 0.15 M NaCl, 3.3 mM CaCl₂ (pH 8.0) for a total of 2 h. The epidermis was placed with the cornified cells down and all basal, spinous and granular cells were scraped off. The sheet of cornified cells was washed twice in 10 mM Tris-HCl, 0.15 M NaCl (pH 8.0). Cornified cells were homogenized in 100 mM Tris-HCl, 0.15 M NaCl, 10 μg/ml phenylmethylsulphonylfluoride (pH 8.0) with a glass homogenizer and incubated at 37 °C for 1 h to remove ribonucleic protein (Smetana, Steele & Busch, 1963). The sample was centrifuged at 13,000 g for 15 min and the precipitate homogenized in 1 M potassium phosphate (pH 7.0) at 37 °C for 1 h to extract proteins derived from keratohyalin granules (Ugel, 1969). After centrifugation at 13,000 g for 15 min, the keratin was extracted from the precipitate in 10 mM Tris-HCl, 8 M urea, 0.1 M β-mercaptoethanol (pH 8.0) (25 ml/g wet tissue) at 37 °C for 4 h. The extracted protein was acidified with 0.1 N HCl and the precipitate formed at pH 6.3 was collected.

In vitro keratin fibre formation

The technique used by Steinert (1975; Steinert et al. 1976) for bovine epidermal and keratin polypeptides was slightly modified. Briefly, the pH 6.3 protein precipitate was dissolved in 6 M urea containing 25 mM β-mercaptoethanol and centrifuged at 25,000 g for 2 h. The supernatant was adjusted to 1 mg protein (measured by the technique of Bradford (1976)) per ml of 6 M urea and was dialysed against 1000 vol. of 5 mM Tris-HCl, 25 mM β-mercaptoethanol (pH 8.0) at 22 °C for 24 h. The sample was divided into 2 portions and β-mercaptoethanol was added to one of them at a final concentration of 100 mM per ml.

Formation of macrofilaments

The protein samples dialysed against 5 mM Tris-HCl were divided into 1-ml aliquots and dialysed in 5 mM Tris-HCl, containing 25 mM β-mercaptoethanol and 1 mM EDTA (pH 8.0) overnight at 22 °C. They were then dialysed against the same buffer without EDTA for an additional 24 h. KCl, NaCl, CaCl₂, MgCl₂, ZnCl₂, CuSO₄, or HgCl₂ (pH 8.0) were added to the buffer at a concentration of 0.3 to 1 mM. After overnight dialysis, the gross changes which occurred were observed directly in test tubes. The samples were also placed on a microscope slide or Petri dish and examined with a Leitz inverted light microscope.

The ions mentioned above were also added to protein samples on a microscope slide at a final concentration of 1 mM per ml. A coverglass was gently placed over the protein solutions.
and fibre formation was observed under a Zeiss photomicroscope using phase-contrast and polarization optics. Photos were taken at x 40 or 100 magnification.

Protein samples isolated and dialysed against 5 mM Tris-HCl, 25 mM β-mercaptoethanol (pH 8.0) were centrifuged at 150,000 g and the precipitate was dissolved in 6 M urea at a concentration of 1 mg protein per ml. It was dialysed again in 5 mM Tris-HCl, 25 mM β-mercaptoethanol (pH 8.0) at 22°C for 24 h, and diluted to approximately 50 µg/ml with the same buffer. One drop of the protein solution with and without 1 mM MgCl₂, CaCl₂, or ZnCl₂ was placed over on carbon-coated ‘stress-free’ grids (Ladd Research Ind. Inc.) and negatively stained with 1% aqueous uranyl acetate without prior fixation.

All samples were examined with a Siemens Elmiskop 1A electron microscope at 80 kV. All photographs were taken at magnifications of 20,000 or 40,000 ×. The magnification was corrected by the use of a grating replica (Ernest F. Fullam, Inc.).

Polyacrylamide gel electrophoresis (PAGE)

Protein samples lyophilized after the second polymerization were dissolved in 6 M urea. They were mixed with a final concentration of 1% SDS, 10 mM sodium phosphate (pH 7.0), 1% β-mercaptoethanol and 15% sucrose and heated for 3 min in a boiling waterbath. The gels (12 cm x 0.5 cm) of 7.5% acrylamide with 0.1% SDS were used and electrophoresis was done for 7 h with 8 mA per tube using a continuous buffer system of Weber & Osborn (1969). The gels were stained with Fast Green by the method of Gorovosky, Carlson & Rosenbaum (1970) and destained in a diffusion destainer (BioRad Model 170). Phosphorylase a (94,000), bovine serum albumin (68,000), and ovalbumin (46,000) were used as standards for the calculation of molecular sizes.

Amino acid analysis

The proteins polymerized for the second time were hydrolysed in constant-boiling distilled 6 N HCl for 24 h under vacuum at 110°C and analysed with a Beckman automatic amino acid analyser.

RESULTS

As reported previously (Shimizu et al. 1974; Inoué, Fukuyama & Epstein, 1976), the isolation of ‘pure’ cornified cells from newborn rat epidermis is not difficult (Fig. 1) without using trypsin or other proteolytic enzymes. Formation of approximately 7- to 8-nm-wide filaments was observed when protein extracted from the isolated cornified cells in Tris-HCl-buffered urea solution containing β-mercaptoethanol was dialysed against 5 mM Tris-HCl (Fig. 2). The length of these filaments differed from one extraction sample to the next. The shorter filaments measured about 0.1 nm, but the longer ones often formed a meshlike network and measurement of their length became impossible. In general, the filaments seemed to become longer after adding increased concentrations of β-mercaptoethanol, as seen by Steinert et al. (1976). When the protein solution was placed on a microscope slide and examined by phase-contrast microscopy, the presence of long and very thin filaments was noted only occasionally (Fig. 4). The filaments showed positive but very weak birefringence under the polarizing microscope. However, the protein solution in test tubes showed no precipitate or turbidity and also was not particularly viscous.

These physical properties changed after dialysis of the protein solution in Tris-HCl buffer containing the various divalent cations. Dialysis against KCl and NaCl did not influence the protein. The protein solution in MgCl₂ or CaCl₂ gave the
appearance of a gel. When the protein solutions were transferred into test tubes, they no longer flowed along the tube walls and this gelation was independent of temperature changes between 4 and 37 °C (Fig. 3). Dialysis of the gelled protein against 5 mM Tris-HCl, pH 8-0, had very little effect on viscosity, while dialysis in 1 mM EDTA overnight resulted in a return to solution. The gelled protein examined by light microscopy demonstrated numerous very long filamentous aggregates with strong birefringence (Fig. 5). Addition of MgCl₂ or CaCl₂ to the protein solution on the microscope slides also produced linear aggregates. The time required for development of the long filaments was usually less than 10 s. By electron microscopy, the filaments clumped into small aggregates, or they assembled side by side and formed wider filaments (Fig. 6). After the gel formation, it became technically difficult to prepare samples for negative staining.

Addition of ZnCl₂, or HgCl₂ to the keratin protein solution at 1 mM concentration produced a clot-type aggregate in the test tubes. Addition of CuSO₄ caused a particulate precipitate which settled to the bottom of the test tube (Fig. 3). Lower concentration of the salts resulted in gelation of keratin, similar to that seen with 1 mM CaCl₂. The ‘clot’ was easily picked up by forceps, fixed, embedded and cut for electron microscopy. Bundles consisting of thin filaments were present, closely resembling tonofilament bundles seen in basal through granular cells of newborn rats. In negatively stained preparations, bundles of filaments also were observed (Fig. 7). They showed tightly packed parallel filaments about 5-7 nm in diameter. Structure within filaments, such as a repeated pattern observed in the paracrystals of actin filaments (Kane, 1976; Harris, Tso & Epstein, 1977), was not observed.

When filament bundle formation was examined by light microscopy, linear aggregates thicker than those seen after the addition of CaCl₂ or MgCl₂ were found (Figs. 8, 9). The tightly packed fibres were not dissolved by addition of EDTA, but solubilized in Tris-HCl-buffered 8 M urea (pH 8-0), containing 25 mM β-mercaptoethanol at 22 °C. Upon dialysing the protein-urea solution in 5 mM Tris-HCl (pH 8-0), with 25 mM β-mercaptoethanol for 24 h at 22 °C, microfilaments 7 to 8 nm in diameter were formed once again.

The polypeptide patterns and the molecular sizes of their microfilaments precipitated by centrifugation at 150000 g for 1 h and of macrofilaments precipitated by CaCl₂ or ZnCl₂ and subsequent centrifugation at 15000 g for 15 min were identical.

Fig. 1. Isolated cornified cells. × 500.

Fig. 2. In vitro polymerized keratin filaments of newborn rat epidermis stained with uranyl acetate on a grid. × 120 000.

Fig. 3. Gross changes observed in keratin solution after addition of 1 mM each of (1) ZnCl₂, (2) MgCl₂, (3) CuSO₄, (4) CaCl₂, and (5) HgCl₂. MgCl₂ and CaCl₂ gelled the solution whereas the other ions formed aggregates.

Fig. 4. Long, thin filaments rarely observed with a polarizing microscope after dialysis of solubilized keratin in 5 mM Tris-HCl containing 25 mM β-mercaptoethanol. × 250.

Fig. 5. Filaments formed after addition of 1 mM CaCl₂ to keratin showing definite birefringence under a polarizing microscope. × 250.
Fig. 6. Negatively stained keratin filaments after exposure to CaCl₂ (A) and MgCl₂ (B). ×120,000.

Fig. 7. Tightly packed filaments formed by addition of 1 mM ZnCl₂ in keratin solution placed on a grid coated with Formvar and carbon. Stained with uranyl acetate. ×200,000.

Fig. 8. Polarizing micrographs of numerous macrofilaments formed in keratin protein after addition of 1 mM ZnCl₂. Rotation of the image from Fig. 8 to Fig. 9 demonstrates changes of filaments exhibiting birefringence. ×250.
Table 1. Amino acid composition of keratin fibre prepared from protein of cornified cells of newborn rat

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Value (mol/100 mol)</th>
</tr>
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<tbody>
<tr>
<td>Lysine</td>
<td>4.1</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.9</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.9</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>8.0</td>
</tr>
<tr>
<td>Threonine</td>
<td>3.5</td>
</tr>
<tr>
<td>Serine</td>
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</tr>
<tr>
<td>Glutamic acid</td>
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<tr>
<td>Proline</td>
<td>0.7</td>
</tr>
<tr>
<td>Glycine</td>
<td>24.8</td>
</tr>
<tr>
<td>Alanine</td>
<td>3.9</td>
</tr>
<tr>
<td>Valine</td>
<td>3.4</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.4</td>
</tr>
<tr>
<td>Isoleucine</td>
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</tr>
<tr>
<td>Leucine</td>
<td>7.1</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.2</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.0</td>
</tr>
<tr>
<td>Half cysteine</td>
<td>0.2</td>
</tr>
</tbody>
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The data are presented as mol/100 mol.

Two polypeptide bands of approximately 68,000 and 60,000 Daltons were detected by SDS-PAGE. Amino acid analysis of the microfilaments is summarized in Table 1.

DISCUSSION

The fibrils assembled in vitro from keratin of newborn rat appeared comparable ultrastructurally to those of bovine epidermis as reported by Steinert (1975; Steinert et al. 1976), although the molecular size of polypeptides isolated from the 2 species is different. A significant difference however, seems to be the formation of macrofibres, and gelation of newborn rat but not bovine keratin after addition of divalent cations.

Metallic ions can bind proteins by interaction at single or multiple sites (Steinhardt & Reynolds, 1969; Gurd & Wilcox, 1956). The molecular sites at which divalent cations are bound to keratin have not been determined, but a large number of negatively charged amino acids occur in keratin (Table 1). Since all divalent ions studied induced the macrofibris, but not KCl and NaCl, it seems probable that the ions form crosslinks between keratin molecules and alter solubility. The linking seems to occur both laterally and longitudinally and the filaments become visible by light microscopy. The present findings are similar to observations made previously with a fibrous protein isolated from bovine brain precipitated with Mg²⁺ (Shelanski, Albert, DeVries & Norton, 1971).

The addition of ZnCl₂, CuSO₄ and HgCl₂ produced wider macrofilaments than those formed with CaCl₂ or MgCl₂. Also, Zn²⁺, Cu²⁺ and Hg²⁺ caused many fibres to form and the precipitates clotted at 1 mM concentration, whereas the protein gelled at 0.3 mM concentration. Both light and electron microscopy indicated that these ions seem to induce additional conformational changes in keratin: the filaments were packed tightly. The metal ligand bonds did not chelate with EDTA, but Tris-HCl-buffered urea solution returned the protein to its initial monomeric conformation and removal of urea and the cations allowed the microfibrils to polymerize again.

SDS gel electrophoresis indicated that the polypeptide patterns of the samples
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obtained with or without prior precipitation with CaCl₂ or ZnCl₂ were identical. This finding differs from the experience of Calissano, Moore & Friesen (1969) who studied calcium crosslinking in purified beef brain protein and observed the formation of 5 separate bands from a hydrodynamically and electrophoretically homogenous protein of 24,000 Daltons.

It is not certain whether or not an ion-keratin complex occurs in the epidermis in vivo and plays a functional role in epidermal cells. Results of the present study and some previously reported information appear to support the view that trace ions are involved in some aspects of epidermal cell functions. Ions may be responsible for the bundling of tonofilaments found in epidermal cells in the basal through granular layers. Observations by Pratley & McQuillen (1973) tend to substantiate this possibility. They found that bundles of tonofilaments of frog epithelium became compact after incubating in Ringer’s solution supplemented with 10 mM CaCl₂. Formation of a dense layer of filaments has also been observed in contractile epidermal cells of the notochord (Cloney, 1966), and convoluted tissue culture cells from a squamous cell carcinoma (Auersperg, 1972).

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Ervin Epstein Jr, M.D. performed the amino acid analyses for this study.

REFERENCES


Epidermal keratin filaments


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