SUBERIMIDATE CROSSLINKING SHOWS THAT A ROD-SHAPED, LOW CYSTINE, HIGH HELIX PROTEIN PREPARED BY LIMITED PROTEOLYSIS OF REDUCED WOOL HAS FOUR PROTEIN CHAINS

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Received 19 July 1978

1. Introduction

The cortical cells in α-keratin fibres like wool are filled with microfibrils, composed of proteins with a low cystine content and a high α-helix content, embedded in a matrix of high cystine, zero helix protein [1]. The microfibril (diam. 7.5 nm) appears to consist of protein subunits regularly arranged in a superhelix [2]. The structure of the subunit is unknown. It has been suggested that it has two [2] or three [2,3] partially α-helical protein chains.

A rod-shaped protein (2 × 20 nm) has been prepared from reduced wool by limited trypsin digestion [4,5]. The protein (merokeratin A_1) has a low cystine and a high helix content, and therefore it may be a large fragment of the microfibril subunit. The molecular weight of intact, undenatured A_1 measured in the ultracentrifuge, was compared [5] with the molecular weight of the component chains of A_1 after denaturation, measured by polyacrylamide gel electrophoresis (PAGE). They were unable to say whether A_1 had three or four component chains because of uncertainties in both measurements.

Using the dimethyl suberimidate crosslinking technique [6] before denaturation and PAGE, we now show that A_1 consists of four chains. Therefore, if A_1 is a fragment of the microfibril subunit, then the subunit itself may also have four chains.

2. Experimental

2.1. *Preparation of the merokeratin [4,5]*

A_1 was prepared at room temperature. Purified Lincoln wool, 1 g, was reduced in 500 ml 0.2 M thioglycollic acid, 0.1 M Na_2HPO_4, pH 10 for 18 h. The reduced wool was rinsed in 0.1 M Na_2HPO_4, pH 8.0, and digested by 0.02 g trypsin dissolved in 20 ml 0.1 M Na_2HPO_4, pH 8.0. Digestion was inhibited after 1 h by 4 ml 0.25% phenylmethylsulphonylfluoride in 50% v/v isopropanol–water. The digest was clarified at 50 000 × *g* for 30 min, the supernatant was diluted to 750 ml with 0.1 M sodium phosphate, pH 6.0 and the solution was exposed to the air for 22 h to reoxidize cysteine groups to cystine. The reoxidized protein was precipitated at pH 4.8. The precipitate was collected by centrifugation at 1000 × *g* for 10 min, redissolved in 5 ml 0.1 M Na_2HPO_4, at pH 8.0, and applied to a Sepharose 6B column. A_1 is the major retarded peak.

2.2. *Suberimidate crosslinking of the merokeratin [6]*

The most concentrated A_1 fraction from the column was diluted to 0.8 mg/ml with 0.1 M Na_2HPO_4, pH 8.0. (Protein concentrations were measured with a Zeiss interferometer.) Eleven 2 ml aliquots of the diluted solution were dialysed against 0.2 M triethanolamine.HCl solutions adjusted to 11 different pH values between 8.2 and 12.4. After dialysis, 2 mg/ml dimethyl suberimidate was added to each aliquot, and the pH values were readjusted to the same 11 values. Crosslinking occurred overnight at room temperature. Another 2 ml aliquot of the diluted solution of A_1 was dialysed against triethanolamine·HCl buffer, at pH 8.5, and allowed to stand overnight at room temperature as a control experiment.

2.3. *Denaturation, PAGE and molecular weight determination [7]*

The 11 solutions of crosslinked A_1 and the solution of uncrosslinked A_1 were dialysed against 0.2 M triethanolamine·HCl, pH 8.5, for 2 h. From a solution of 8 M urea, 1% sodium dodecyl sulphate (SDS), 1% 2-mercaptoethanol in distilled water 0.5 ml was added to 0.5 ml of each of the A_1 solutions, and the mixtures were incubated for 6 h at 45°C. After incubation, 20% glycerol and 0.02% bromophenol blue were added to the solutions. From each solution 50 μl was applied to gels prepared using 0.05 M NaH_2PO_4, 0.075 M Na_2HPO_4, pH 7.2, buffer containing 0.1% SDS, 5% acrylamide, 0.133% *N,N'*-methylene bisacrylamide polymerized by 0.073% ammonium persulphate and 0.11% *N,N,N',N'*-tetramethylethylene diamine. Electrophoresis was carried out using a constant current of 9 mA/gel until the dye front approached the bottom of the tube. The electrophoresis buffer was 0.1 M sodium borate, 0.1 M sodium acetate, 0.1% SDS, pH 8.5. The gels were stained for 30 min at 35°C with 0.25% Coomassie brilliant blue dissolved in 20% trichloroacetic acid, and destained electrolytically in 10% acetic acid in the presence of ion-exchange resin AG 501-X8 (Bio-Rad).

To determine the molecular weight of the uncrosslinked and crosslinked chains from A_1, alcohol dehydrogenase (yeast) was crosslinked in the same way as A_1, and a calibration curve was obtained by plotting the mobilities of the uncrosslinked and crosslinked chains from alcohol dehydrogenase and the mobilities of the chains from ovalbumin, pepsin, α-chymotrypsinogen-A, lysozyme, and chymotrypsin, after they had been denatured and electrophoresed in parallel runs with uncrosslinked and crosslinked A_1, against the logarithm of their molecular weights.

3. Results and discussion

When the merokeratin A_1, not treated with dimethyl suberimidate, is denatured to dissociate it into its component chains, and examined by PAGE on 5% gels there are three bands (fig.1). The molecular weights calculated from the positions of the peaks suggest that the two less intense bands are dimers and trimers of the protein chains in the major band. These small amounts of dimer and trimer may be caused by

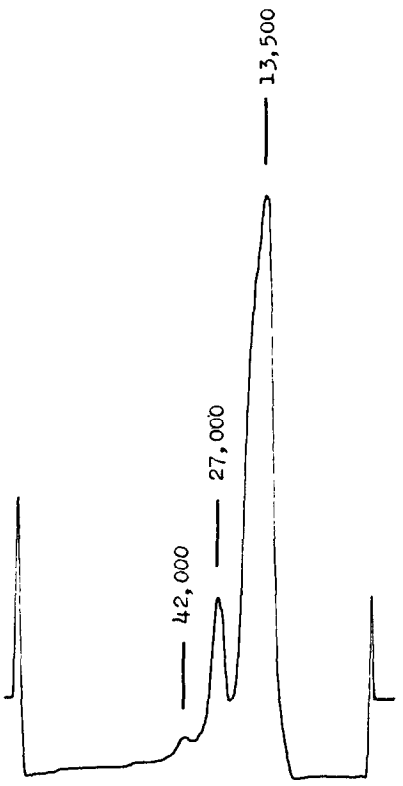

Fig.1. Uncrosslinked merokeratin A_1 denatured and examined by PAGE. Densitometer scan of the gel showing molecular weights calculated from the positions of the peaks.

lanthionine, lysinoalanine or iso-peptide crosslinks between single chains. The crosslinks may be present in the original wool or formed during the preparation of A_1 [8].

The major peak in fig.1 has a shoulder on its heavy side. PAGE of denatured A_1 on 15% gels (not shown) resolves the major peak into two peaks, a larger one (mol. wt 13 500) and a smaller one (mol. wt 15 500). Thus the dimer may consist of three species, mainly mol. wt 27 000 but with smaller amounts of mol. wt 29 000 and 31 000. As expected, the technique did not resolve dimers with these not very different molecular weights. Nor did it resolve the four possible trimers.

Figure 2 is typical of the patterns obtained when A_1 is crosslinked with dimethyl suberimidate at pH values between 9.0 and 11.0, and then denatured and

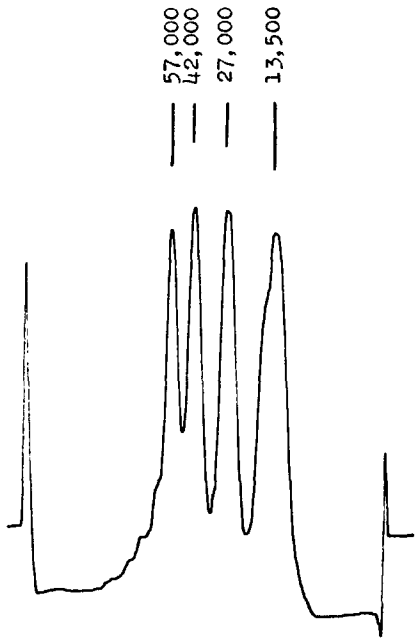

Fig.2. Merokeratin A_1 crosslinked by dimethyl suberimidate, at pH 9.9, and denatured and examined by PAGE: molecular weights calculated from the positions of the peaks.

examined by PAGE. Dimethyl suberimidate is a bifunctional reagent which can react with two lysine residues. When it reacts with a multichain protein it crosslinks chains in the same multichain aggregate much faster than it crosslinks chains in different aggregates. When four-chain enzymes are crosslinked, denatured and examined by PAGE, patterns like fig.2 are obtained: single chains, dimers and trimers are usually present as well as tetramers [6]. The crosslinking reaction is incomplete probably because there are two reactions competing for the methyl imidate groups:

(i) Reaction with the amine groups of lysine residues.
(ii) Hydrolysis by water [9].

If one methyl imidate group in a dimethyl suberimidate molecule reacts with a lysine residue and the other is hydrolysed, then this prevents that lysine residue from taking parts in a crosslink.

When A_1 was crosslinked below pH 9.0, the dimer, trimer and tetramer peaks were smaller than in fig.2 because the lower pH values favour the hydrolysis of the methyl imidate groups over their reaction with lysine residues [9]. When A_1 was crosslinked above pH 11.0, the dimer, trimer and tetramer peaks were again smaller than in fig.2. This suggests that A_1 dissociates into its component chains above pH 11.0.

The mol. wt determined from the positions of the peaks in fig.2 confirm that they are due to A_1 single chains, dimers, trimers and tetramers. The tetramer mol. wt 57 000 is consistent with, but much more precise than, the molecular weight of intact, undenatured A_1 measured in the ultracentrifuge: 50 000–60 000 [4].

These experiments prove that the merokeratin A_1 has four protein chains. The composition and the α-helix content of A_1 and of the microfibril protein are similar. Therefore the experiments imply that the microfibril subunit may also have four protein chains. In contrast, the subunit of the tonofilaments (also diam. 7.5 nm) in mammalian epidermis has three chains [10], and a merokeratin prepared by limited trypsin digestion of epidermal keratin also has three chains [11].

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