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Location of a High-sulphur Protein in Developing Wool Follicles Using Peroxidase-labelled Antibody

R. Frater

Division of Protein Chemistry, CSIRO, Parkville, Vic. 3052.

Abstract

Antiserum to a high-sulphur protein (SCMK-B2) from wool was purified to obtain the immunoglobin G fraction, and this was labelled with horse-radish peroxidase. The labelled antibody was then applied to sections of sheep skin embedded in glycol methacrylate in order to locate the distribution of high-sulphur protein within the lower hair shaft. Regions containing the highest concentration of high-sulphur protein were the cuticle of the hair shaft and the keratogenous zone of the cortex.

Introduction

The disulphide-linked proteins which comprise the entire structure of hair keratin have been studied extensively for many years. Because of the insoluble nature of hair keratin these studies are made possible only by preliminary splitting of the disulphide bonds, generally by reduction and -SH substitution. Reaction with iodoacetate thus forms the *S*-carboxymethyl (SCM) derivatives (O'Donnell and Thompson 1962).

The proteins isolated in this way can be separated into three main classes termed low-sulphur, high-sulphur and high-tyrosine proteins. For a review of the methods used and the characterization of the proteins, see Fraser *et al.* (1972). More recently the complete amino acid sequences have been determined for several purified high-sulphur proteins (Haylett and Swart 1969; Elleman 1972; Dopheide 1973).

This approach to keratin chemistry, however, tells us nothing about the histological location of the various protein types within the native fibre, or of their possible role in determining hair fibre characteristics. The major difficulty in locating the various protein types histologically is in finding a suitable specific marker for each type which can be visualized at the microscopic level. The most specific method is visualization of the proteins (as antigens) using labelled antibodies.

In this way Kemp and Rogers (1970), using fluorescent-labelled antibody to guinea-pig hair follicle protein, were able to demonstrate synthesis of low-sulphur protein in the upper bulb region of the follicle. These workers, however, were not able to establish the region of synthesis of high-sulphur proteins. In the present study, antiserum was prepared in rabbits to a purified high-sulphur protein (SCMK-B2, see Elleman 1972) obtained from wool, and following separation of the immunoglobin G (IgG) fraction, the antiserum was labelled with horse-radish peroxidase. The labelled antibody was then used to locate high-sulphur protein in sections of sheep skin embedded in glycol methacrylate.

One problem encountered in preparing antisera to purified SCM-wool proteins is that cross reaction is also found against all other SCM-wool proteins and also against non-keratin SCM-proteins (Frater 1968). It was also found that the reaction of this antiserum could be made specific by inclusion of potassium thioglycolate (Frater 1968) or SCM-cysteine (Kemp and Rogers 1970) in the diffusion medium. Satisfactory specificity of the antiserum could also be obtained by absorption with polymerized SCM-albumin as will be discussed later in this paper.

Materials and Methods

Materials

Horse-radish peroxidase (RZ 3) was obtained from Sigma Chemical Co. (St Louis, U.S.A.). The specially purified tributyl phosphine was kindly supplied by Dr J. A. Maclaren of this laboratory, and the glycol methacrylate was obtained from Medos, Australia. Freund's complete adjuvant was from the Commonwealth Serum Laboratories, Australia, and the ion-exchanger used in the preparation of IgG fractions was DE 52 from Whatman. The purified high-sulphur protein fraction, SCMK-B2, obtained from Lincoln wool (Elleman 1972) was a gift from Dr T. C. Elleman of this laboratory, and the low-sulphur protein (component 8) was also prepared from Lincoln wool by the method of Thompson and O'Donnell (1965). Bovine serum albumin (Cohn fraction V) was obtained from the Commonwealth Serum Laboratories, Australia, and converted to the SCM derivative (O'Donnell and Thompson 1964).

Preparation and Labelling of Antibodies

An aqueous solution of the protein (2 mg/ml) was emulsified with an equal volume of Freund's complete adjuvant, and 1 ml of the mixture was injected into three rabbits, using multiple intradermal injections. After 3 weeks the injections were repeated, and 3 weeks after this the animals were bled and the serum tested for the presence of antibodies by double immunodiffusion in 0·5% agarose gels. The serum selected for the isolation of IgG had a titre of 1 : 10 as determined by double immunodiffusion.

The γ-globulin fraction from the serum was obtained by precipitation with an equal volume of saturated ammonium sulphate at 5°C, followed by washing of the precipitate with cold 50% saturated ammonium sulphate. The precipitate was dissolved in a small volume of water and dialysed at 5°C first against distilled water, and then against 0·0175 M phosphate buffer, pH 8·0. The protein concentration was adjusted to 3% by dilution with buffer, and a 5-ml sample applied to a DEAE column (0·9 by 20 cm) equilibrated with the same buffer. On elution with the buffer, the IgG fraction in the first 50 ml was collected, dialysed against distilled water at 5°C and freeze-dried. Generally 50–60 mg of protein per 5 ml of sample loaded was obtained. The IgG was then coupled to horse-radish peroxidase (10 mg of peroxidase plus 5 mg IgG) by the method of Avrameas and Ternynck (1971). For application to embedded tissue sections, the labelled antibody was diluted with phosphate buffered saline to a final antibody concentration of 1 mg/ml.

Tissue Processing

Samples of Lincoln sheep skin, taken with a skin biopsy punch, were fixed in cold TCA–ethanol (5% trichloracetic acid in 90% ethanol), and, following equilibration with 80, 95 and 100% (v/v) glycol methacrylate monomer, the samples were finally equilibrated and polymerized in the polymer mixture (Feder and O'Brien 1968). Tissue sections longitudinal to the hair shaft were cut at 4 μm using a Cambridge rocking microtome adapted for use with a glass knife.

Reduction and Alkylation of Tissue Sections

Because of the lengthy embedding process, it was assumed that some oxidation of thiol groups to disulphide may have occurred in the thiol-rich region of the lower hair shaft and bulb. To reverse this possible reaction, and also to convert pre-existing disulphide to SCM derivatives the sections were reduced and alkylated in the one step process described by Maclaren (1971). This process would also convert disulphide-bonded proteins in the upper hair shaft to the SCM derivatives.

The reaction mixture, slightly modified from that described by Maclaren (1971), contained 0·25 ml tributyl phosphine, 47 mg chloracetic acid and 60 mg diethanolamine (final pH 8·0), made to a final

volume of 5 ml with 80% (v/v) *n*-propanol. Sections were kept in this mixture for 2 days at room temperature, after which they were collected onto slides, rinsed free of reagents with 80% (v/v) *n*-propanol and allowed to dry. Brief heating to 100°C as described by Feder and O'Brien (1968) provided firm attachment of the sections for subsequent exposure to antibodies and washing reagents.

Absorption of Antibody

To reduce possible cross reactions of the antibody to other SCM proteins, the labelled antibody was absorbed with polymerized SCM-bovine serum albumin (SCM-BSA) prepared by treatment of SCM-BSA with ethyl chloro-formate (Avrameas and Ternynck 1967). To employ the absorbent, 50 mg of polymer was shaken with 0·5 ml of diluted antibody for 24 h in the cold, and the supernatant was used after centrifugation.

Staining of Skin Sections

Several sections were covered with a few drops of the labelled antibody, and incubated at room temperature for 40 min, after which the slides were rinsed with two changes of phosphate buffered saline, with shaking for 10 min each time. Control incubations were also included, the labelled IgG being prepared from normal rabbit serum.

After rinsing, the slides were incubated for 30 min with shaking in a freshly made solution of 4-chloro-1-naphthol (Koch–Light) in 0·001% hydrogen peroxide (Nakane 1971). The slides were then rinsed briefly with distilled water and mounted with a drop of 50% glycerol containing 5% acetic acid. They were then examined and photographed using a Wild M 20 microscope.

Results

Specificity of Antisera

When antiserum to SCMK-B2 was tested by immunodiffusion, a weak positive reaction was still obtained against SCM-low-sulphur protein even in the presence of potassium thioglycollate or SCM-cysteine. By absorption of the antiserum with polymerized SCM-BSA, the non-specific reaction to SCM-low-sulphur protein was eliminated, and a positive reaction was obtained only against SCMK-B2 (Fig. 1). The absorbed antiserum also cross reacted with all other wool proteins belonging to the high-sulphur group (Frater, unpublished data). As an added precaution against non-specific reaction, the peroxidase-labelled IgG preparation also contained 0·05 M sodium SCM-cysteine.

Staining of Sections

Fig. 2 shows the result obtained when sections were stained with the labelled antibody to the high-sulphur protein. The section contained one almost complete hair shaft comprising most of the lower hair follicle, the keratogenous zone, and a short length of fully hardened hair. Staining was observed in the cuticle of the hair shaft beginning at about the level of the keratogenous zone and continuing to the end of the shaft. The displacement of the upper shaft from the plane of the section made it possible to see that the cuticle of the inner root sheath was also stained by the labelled antibody.

Within the cortex of the hair shaft there was little or no labelling in the follicle, and a progressive labelling beyond this which reached maximum intensity in the keratogenous zone. Beyond this zone the intensity of labelling decreased. Several sections were stained in this way in three different experiments, and identical results were obtained in all cases. Non-specific staining was minimal in this experiment, as peroxidase-labelled normal rabbit IgG produced no staining in similar skin sections. A blocking control was carried out by pre-incubating sections with unlabelled IgG to

SCMK-B2, followed by treatment with labelled IgG to SCMK-B2 as described earlier. No staining was observed in sections treated in this way.

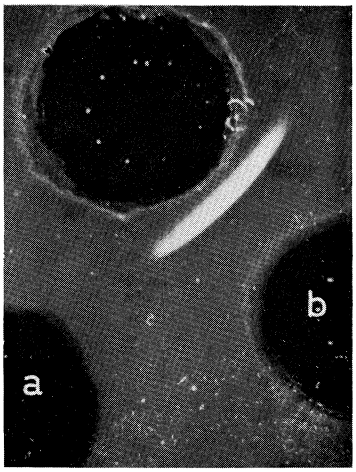

Fig. 1. Double immunodiffusion test in 0·5% agarose, buffered with 0·03 M barbital, pH 7·0, and also containing 0·1 M SCM-cysteine. Top well (unlabelled) contains rabbit antiserum to a high-sulphur protein (SCMK-B2), absorbed with polymerized SCM-BSA. The other wells contain (*a*) 1 mg/ml low-sulphur protein and (*b*) 1 mg/ml SCMK-B2. The gel was developed at room temperature for 24 h and photographed unstained using dark-field illumination.

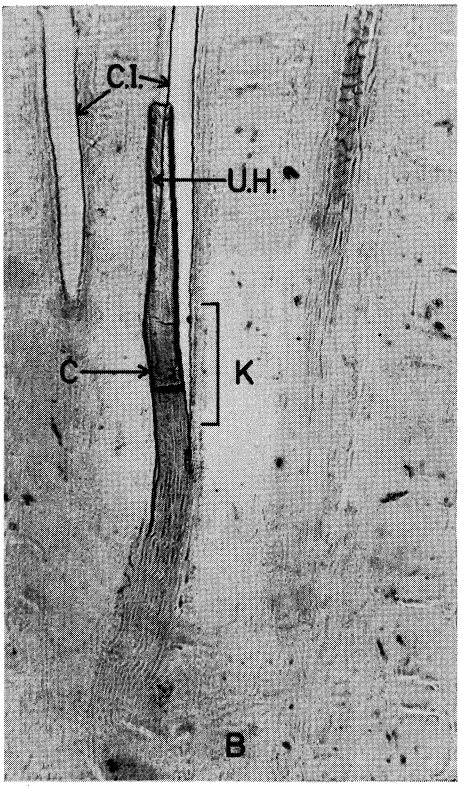

Fig. 2. Section of embedded sheep skin, reacted with absorbed peroxidase-coupled rabbit antiserum IgG to SCMK-B2. *B*, Hair bulb (follicle); *K*, keratogenous zone; *C*, hair shaft cuticle; *U.H.*, upper (hardened) hair shaft; *C.I.*, cuticle of inner root sheath.

Discussion

The conclusion from these results, concerning the distribution of high-sulphur protein within the hair shaft, confirms the previous suggestion obtained by amino

acid analysis of separated cuticle (Bradbury *et al.* 1970) that the cuticle may be rich in this group of proteins. The lack of staining in the keratinized upper portion of the hair may be attributed to the compactness of keratin in this region and consequent poor antibody penetration. A similar finding was reported by Kemp and Rogers (1970). The lack of staining in the bulb region, coupled with the progressive increase towards a maximum at the keratogenous zone, provides supporting evidence for the postulated synthesis of high-sulphur proteins within the keratogenous zone (Downes *et al.* 1963; Gillespie 1965).

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