Cell-Free Biosynthesis of Wool Keratin Proteins

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The polyribosome complements in wool roots (Wilkinson, 1970) and in hair follicles (Steinert & Rogers, 1971a) are broadly similar, being characterized by a polydisperse group of 'light' polyribosomes that sediment in several discrete bands and a group of 'heavy' polyribosomes that sediment as a broad band on sucrose-density-gradient centrifugation.

The full complement of both heavy and light polyribosomes present in a whole hair-follicle homogenate from guinea pig synthesizes protein in a reconstituted cell-free system (Steinert & Rogers, 1971a). Fractionation of the resultant protein by pH precipitation in the presence of carrier yielded labelled low-sulphur keratin proteins (Steinert & Rogers, 1971b). This group of proteins is one of the two found in hair and wool keratin: a high-molecular-weight low-sulphur microfibrillar fraction has been ascribed a molecular weight of 40 000–50 000 and represents 50–60% of wool protein (O'Donnell & Woods, 1956), and a low-molecular-weight high-sulphur matrix fraction is composed of proteins of widely differing molecular weights (14 000–30 000) and represents 20–30% of wool protein (Lindley, Gillespie & Haylett, 1967).

The present study shows that the heavy polyribosomes are responsible for the synthesis of the low-sulphur proteins of the wool root and that at least some of the high-sulphur proteins are made on the light polyribosomes (cf. Wilkinson, 1970).

Wool-root homogenates were obtained as described by Wilkinson (1970), except that 2-mercaptoethanol in the extracting buffer was replaced by 1 mM-dithiothreitol. Cell supernatant fractions (12 ml) were layered over discontinuous sucrose density gradients consisting of 1 ml of 17% (w/v) sucrose, 1 ml of 40% (w/v) sucrose and 10 ml of 2 M-sucrose, all in extraction buffer modified by decreasing the concentration of potassium chloride to 0.1 M [i.e. 0.02 M-triethanolamine hydrochloride–NaOH (pH 7.8)–0.2 M-sucrose–0.1 M-KCl–5 mM-magnesium acetate–1 mM-dithiothreitol]. The gradients were centrifuged in an SW 25.1 rotor of a Beckman L4 ultracentrifuge for 2 h at 4°C at 23 000g (r_{av} , 9.1 cm). Zones that scattered light were observed by using an ISCO model D fractionator, and a 2 ml sample was withdrawn from

above the 2 M-sucrose layer by side puncture. The fraction was dialysed against the gradient buffer overnight and the 'total' polyribosome preparation was used directly in cell-free systems. Preparations containing predominantly heavy polyribosomes were obtained as described above, except that the 40%-sucrose layer was increased to 4 ml, the gradients were centrifuged for 2 h at 4°C at 15 000g (r_{av} , 9.1 cm) and only 1 ml fractions were taken by side puncture.

A pH 5 enzyme preparation was obtained essentially by the method of Falvey & Staehelin (1970), by using the following low-ionic-strength buffer: 0.01 M-triethanolamine hydrochloride–NaOH (pH 7.8)–0.2 M-sucrose–5 mM-magnesium acetate–1 mM-dithiothreitol. The precipitate was finally dissolved in gradient buffer and stored in small portions at –80°C until required. This method of preparing pH 5 enzymes has the advantage that tRNA is satisfactorily reclaimed and is sufficient for cell-free biosynthesis. The low-ionic-strength buffer limits contamination of the enzyme preparation by low-sulphur proteins, which pellet with the ribonucleo-protein during centrifugation.

The cell-free system contained, in 1 ml: 50 μ l of energy mixture in water, containing ATP (1 μ mol), GTP (0.25 μ mol), phosphoenolpyruvate (5 μ mol); 20 μ l of amino acid mixture in water, containing 1 μ g each of 19 L-amino acids (excluding valine); 20 μ l of [^{14} C]valine in water (1.25 μ Ci, 260 mCi/mmol); 10 μ l of pyruvate kinase (100 μ g); 100 μ l of gradient buffer (at twice the usual concentrations); 600 μ l of polyribosomes (0.6–1.0 mg); 200 μ l of pH 5 enzyme (2.4 mg of protein).

Incubations were for 1 h at 37°C and were terminated by rapid cooling in an ice bath. A fraction (0.1 ml) was prepared for radioactivity counting in a Beckman LS 200 liquid-scintillation spectrometer by the method of Mans & Novelli (1961). To the remainder was added 0.5 ml of freshly extracted wool-root protein (2 mg) in 0.01 M-tris–1 mM-EDTA–8 M-urea, pH 9.4; 1.5 ml of the urea buffer was added, followed by urea to make the final concentration 8 M. The mixture was shaken for 1 h at 40°C, and the proteins were reduced with potassium thioglycollate (0.2 M) and alkylated with iodoacetic acid until the nitroprusside test was

negative. A slight excess of 2-mercaptoethanol was then added, and the mixture was dialysed against water for 48 h, freeze-dried and dissolved in 0.2 ml of the urea buffer (Fraser, 1969).

Horizontal starch-gel electrophoretograms were run as described by Thompson & O'Donnell (1964), the top slice was stained with Nigrosine and Amido Black (Fraser, 1969) and the remainder of the gel was cut into 40 pieces, which were each placed in 2 ml of water in a scintillation vial. The gel pieces were broken up with a nylon rod and sonicated for a few seconds until the suspension cleared. Then 15 ml of a xylene-based scintillation fluid containing Triton X-100 was added, and the mixture was thoroughly shaken and counted for radioactivity at 63% efficiency (Wilkinson, 1971).

Fig. 1 illustrates the radioactivity profiles of [^{14}C]valine incorporated into protein in cell-free systems with wool-root total polyribosomes and with heavy polyribosomes. A diagrammatic representation of a stained slice of the electrophoretogram is shown as a reference. Protein bands are resolved particularly well, the characteristic proteins of the low-sulphur fraction being evident from the origin to midway along the gel, and the high-sulphur proteins occur from midway to the gel front (Thompson & O'Donnell, 1964; Fraser, 1969). Starch-gel electrophoresis was used in the present study because of the particular success achieved with this method, because the combined effect of molecular sieving and electrophoretic separation in the presence of 8M-urea would tend to limit non-specific aggregation, and because of the wealth of information available on the behaviour of wool and wool-root proteins on starch-gel electrophoresis.

The proteins synthesized by the total polyribosome preparations include both low- and high-sulphur groups, whereas those synthesized by preparations containing predominantly heavy polyribosomes are almost exclusively low-sulphur proteins. The synthesis of low-sulphur proteins in the present study supports the work of Steinert & Rogers (1971b), but, by fractionating the polyribosomes before cell-free protein synthesis rather than fractionating the protein product, the role of the polyribosome is now defined.

It has not yet been possible to isolate light polyribosomes sufficiently free of heavy polyribosomes or of artificially light material resulting from shear of heavy polyribosomes to have full confidence in cell-free-system product analysis. The contribution to the protein product by light polyribosomes in cell-free systems of total polyribosomes is apparent from Fig. 1 and indicates their association with high-sulphur protein synthesis. These proteins are very heterogenous in size and amino acid composition (Crewther, Fraser, Lennox & Lindley, 1965; Fraser, 1969). They can contain as much as 25% of

Fig. 1. Electrophoretic fractionation on starch gel of ^{14}C -labelled proteins synthesized in cell-free systems containing wool-root total polyribosomes (—) and predominantly heavy polyribosomes (---). The starch-gel electrophoretogram of these proteins with added carrier wool-root protein is shown for reference. The low-sulphur protein bands are numbered as described by O'Donnell & Thompson (1964). The pattern shows that band 7 is divided into two separate proteins and an additional protein is separated between bands 7 and 8. This improved resolution was obtained by using a modified gel buffer system (Fraser, 1969, and personal communication).

cysteine residues (I. E. B. Fraser, unpublished work), and the cysteine residues can occur as a repeating sequence (H. Lindley, personal communication). Full definition of the role of light polyribosomes in the synthesis of this group of unusual proteins is now highly desirable.

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