Relating Self-assembly to Spatio-temporal Keratin Expression in the Wool Follicle

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Abstract

Two of the present authors have presented a model for macrofibril template self-assembly which is based on a concerted process of sudden, delayed, short filament formation with immediate phase transfer to a lyotropic liquid crystal (mesophase). In recent years, information on the spatio-temporal patterns of keratin protein expression within wool follicles has come to hand, and we here report some recently acquired important information in this area. If the “mesophase model” is to remain credible it must be compatible with the spatio-temporal patterns of keratin expression. This paper explores the match between the mesophase model and patterns of protein expression, and concludes that they are broadly consistent. At present the model does not extend to keratin associated protein (KAP) expression. The model is also particularly successful in explaining macrofibril structural features in different cell types.

Key Words: Keratin, Expression, Macrofibril, Wool, Hair

1. Introduction

The cortex cells central to keratin fibres are composed almost entirely of trichocyte keratin intermediate filaments (IFs), and their associated proteins (KAPs), within ordered and interlinked bundles (macrofibrils) oriented with the fibre axis. Cross-linking and other physical changes occurring during the keratinisation, or hardening, process are among the final stages of a remarkable self-assembly process. As newly dividing cells from thefollicle base form what becomes the fibre cortex and move up the follicle, various keratins are successively expressed and assemble into macrofibrils. Macrofibril template self-assembly appears to occur via a concerted filament growth and mesophase (lyotropic liquid crystal) separation process driven by IF assembly. Our earlier works[1-3] focussed on the evidence for this model, primarily from ultrastructural data[4,5] (Fig.1), and the supporting thermodynamic theories[6,7] to describe mesophase formation in the initial form of tactoids, and suggested also a model for the filament formation which is akin to statistical mechanical models of other protein transitions[8,9].

Here we review key parameters of the mesophase model, including the formation of unit-length filaments (ULFs, the axial sub-unit of IFs), and the roles of keratin molecule head and tail groups in IF formation, and then discuss the model in the context of the spatio-temporal pattern of protein expression in

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Fig. 1 Transmission electron micrographs of developing macrofibrils. A. Macrofibril (Zone C – see Fig. 2 for zone definition) coalescing from two liquid crystal tactoids. B. Tactoids form in Zone B either associated with membranes or isolated in the cytoplasm. C. A desmosome cell junction forms an anchor for early forming IFs. D. Transverse section of early tactoid showing IF spacing.
wool follicles. We conclude by exploring in Section 7 how the timing of expression events in ortho-, meso- and para-cortex cells relates to their final macrofibril architecture, adding to comments in a previous publication, and highlight areas where further investigation will be required.

2. Spatio-temporal protein expression in the follicle

Langbein and colleagues[10,11] have played a pivotal role in elucidating protein expression in all cell types of human scalp and beard follicles, using both in situ hybridisation and immuno-labelling microscopy. Yu and colleagues[12,13] confirmed from RNA patterns that those expression patterns are highly conserved in wool, (although an additional minor Type II keratin, K87, is expressed in wool). Most importantly, for understanding wool macrofibril development, RNA and proteins were localised with reference to landmark morphology in the follicle such as the top of the dermal papilla and the point at which Henle’s layer of the inner root sheath hardens, just above the bulb. This gives us enough information to match protein expression to developing fibre ultrastructure as defined in earlier work[14] (Fig. 2). Keratin IFs are composed of heterodimers (of Type I and Type II keratin molecules) and we are particularly interested in what partners are available during macrofibril development.

The first Type I keratin expressed is K35, which within a few cell lengths is joined by K31. The latter, together with initial Type II keratin K85, dominates the expression pattern through much of the bulb region. Just above the top of the bulb (where Henle’s layer hardens) Type I K38 is expressed, typically on one side of the cortex only. Expression of Type I K33a&b commences at about this point, matched with Type II K83 and K81. A little later, Type I K34 expression begins. We do not here discuss the minor components K37 and K39. Specific combinations of Type I and Type II keratins are particularly relevant to the mesophase model. Head group size is an important parameter in that model, and the role played by short heads (56 amino acid residues, K31, K33a&b, one form of K34) versus long heads (97, K35; 104, K38; 72[15] or possibly 98, in alternative forms of K34) is crucial.

3. ULF formation

The process by which keratin proteins assemble into the monomers of IFs, the 32-chain unit length filament (ULF), is well described elsewhere[16,17]. In short, a Type I/Type II combination forms a largely coiled-coil heterodimer, two of which then assemble anti-parallel to form a tetramer, in which the dimers may be displaced longitudinally in one of three ways, stabilized by electrostatic interactions (the A11, A22, and A12 overlap schemes[18]). Eight such tetramers then assemble into a loose cylindrical structure, the ULF. ULFs may, or may not, then proceed to polymerise (or “anneal” in the usual parlance).

In a recent thermodynamic study of the ULF formation process, using isothermal titration calorimetry, Ishii and colleagues[19] found that only the tetramer formation step was significantly exothermic, and that the other levels of association were stabilized by entropy changes in the water released from the lower level of association. These findings are consistent with our previous theoretical conclusions[1], but we underestimated the importance of such water entropy terms in stabilizing the structure of the ULF. The finding that tetramer formation is exothermic is vital to our model (see section 5 below).

4. ULF polymerization and head/tail involvement

It is well established[18] that in the formation of other types of IFs the head and tail groups of the ULFs play a critical role in determining if filament formation proceeds.

In our model we focus on head groups because they differ markedly from those of other types of IF-forming proteins, making them the obvious major variable in trichokeratin selection for filament assembly. It is fundamental to our model that ULFs formed from short-head Type I proteins (K31, 33a&b) do not proceed to filament formation, until activated by the inclusion into the ULF of a long-head Type I, which stimulates an axial shift in the ULF, leading to polymerisation.

Although we have no explicit information on what particular structures of the ULF may be involved, it is convenient to explain...
this model in terms of an A12 assembly mode[18] (ie, fully overlapped dimers in the tetramer) compared with a displaced A11 or A22 mode. The former represents a “blocky” ULF, whereas the latter presents as a “shaggy-ended” ULF (Fig. 3). Shaggy-ended ULFs interdigitate to form a stabilised and growing filament, each shaggy end creating an axial displacement in the next ULF it adds to the chain. The stimulus for the initial axial shift occurs when a large-head Type I protein is incorporated into a blocky ULF and comes about from the steric crowding of the head and tail pendant groups in the close-packed A12 mode. The loss of pendant-group conformational entropy in the A12 structure may be just tolerable when the head groups are small, but when they get bigger it is energetically favourable to displace the sub-units to relieve entropic constraints, possibly at the expense of hydrophobic entropy losses in the water surrounding the displaced ULF. Of course, this water entropy loss occurs only in the two end units of a possibly long chain, but, assuming dimer-dimer interactions are involved, we gain enthalpic energy as each ULF is added. Many aspects of the process described here are shared with very recent theories of filament formation by other types of IF, especially vimentin[20].

5. Modelling filament formation

We address this topic according to a particular version of equilibrium polymerisation theory, capable of explaining “sudden event” polymerisations exhibiting floor or ceiling temperatures[21]. In the terminology of Tobolsky and Eisenberg[22], we apply Case 1a, which involves two equilibrium constants, denoted $K$ and $K_r$, relating respectively to initiation and addition. If a ceiling temperature exists, the addition reaction must be exothermic. In the context of ULF polymerization, we assume that at biological temperature, which in our case will be generally between 35°C and 37°C, undisplaced ULFs are above their ceiling temperature and thus thermally depolymerised. The observation of K31/K83 filaments being formed at much lower temperatures in vitro[23] is consistent with this hypothesis. In this model, the ceiling temperature is also dependent on the initial concentrations of both the monomer and the initiator. The latter is a ULF in a displaced activated form ($U^*$ below, shaggy form), which is capable of reacting with an undisplaced ULF (U, blocky form) to form a displaced dimer, with on-going propagation.

\[
\text{Activation} \quad U \leftrightarrow *U^* \quad \text{Equilibrium constant} K
\]

(based on incorporation of a long-head Type I keratin)

\[
\text{Addition} \quad *U^* + U \leftrightarrow *U_{n+1}^* \quad \text{Equilibrium constant} K_r
\]

\[
\text{Equilibration} \quad *U_{n}^* + *U_{m}^* \leftrightarrow *U_{n+m}^* \quad K_r/K
\]

Our model thus proposes that there will be no IF (polymer) species formed until the ULF concentration reaches a level at which, given the prevailing initiator concentration, the ceiling temperature almost reaches biological temperature. At this point the initial polymer formed will be of low degree of polymerisation, as is in fact observed (at the precise ceiling temperature, the degree of polymerisation will be much higher). Differences in the spatial expression patterns and availability of IF-forming initiators may be a key factor in the creation of macrofibrils associated with different cell types as in orthocortex and paracortex. Tactoid formation will be delayed in cells with lower initiator or ULF concentrations. We are continuing to review this model, many aspects of which would be amenable to test given the availability of recombinant proteins.

6. Connecting IF formation to protein expression

Large numbers of tactoids first appear within the developing cortex cells in the upper half (Zone B) of the follicle bulb (Fig. 1B),

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This text is a continuation of the previous discussion on keratins and ULF activation states. It further explores the assembly modes of keratin dimers within tetramers and their implications on filament formation. The text introduces a model for understanding filament formation through equilibrium polymerisation theory, highlighting the role of specific keratin proteins and the influence of various concentrations on filament stability and growth. The discussion then extends to the connection between IF formation and protein expression, emphasizing the spatial and temporal aspects of tactoid appearance in developing cortex cells and the potential implications for understanding the biological processes involved.
and the most populous keratin species at that stage are Type I K31 and Type II K85. We believe that from the beginning of this Zone (level with or slightly below the tip of the dermal papilla), K31/K85 expression in cortex cells builds up a high concentration of ULFs based on this pairing. Once a high concentration is achieved, and given ULFs themselves are anisometric, with a rod axial ratio ~5-6, any further lengthening of the ULFs into short IFs will quickly result in the formation of a concentrated oriented phase, the mesophase, appearing initially as spindle-shaped tactoids (if the mesophase is nematic). The details of how these processes occur are covered in an earlier publication[1]. Using antibody probes raised against K31 it is possible to see the relatively sudden appearance of dense sub-cellular structures after a period of more diffuse signal through the cytoplasm (Fig. 4).

The relatively sudden onset of concentrated mesophase structures in cortex cells (either within the cytoplasm or associated with desmosomes) is in contrast with events at the same point in the Henle’s and Huxley’s cells of the Inner Root Sheath, where a loose network of filaments reminiscent of a cytoskeleton forms, and becomes gradually more dense as it develops.

In the cortex this gradual build-up of a cytoskeleton-like network is not observed and our model suggests that this is due to suppression of large-scale filament formation until initiated by the build-up of an initiator species following which mesophase formation is stimulated. K35, because of its unusually large head group (for a Type I keratin) is a possible candidate for an initiator role, as it is possible for protein interchange to occur with K31, by disassembly and reassembly processes. Moreover, as protein expression continues into Zone C above the bulb, desmosomes (see Fig 1), with which K35 IFs are likely associated, de-construct, and K35 may be released in this process. K38 is another potential large-head Type I candidate. It is expressed only in the orthocortex, its expression increasing as that of K35 declines across the cortex. It is possible that K38 moderates development of orthocortex macrofibrils which may account for observations that orthocortex develops later and keratinises later than paracortex[24] (Fig. 4A inset).

At a higher level in the follicle (mid-Zone C), another grouping of abundant keratins begin to strongly express, with K33 isoforms being the primary Type I proteins.

K33a and K33b keratins likely form heterodimers with K81 or K83. Activation and regulation of blocky-type ULFs of these species to shaggy ULFs and then filaments may be mediated by a long-head version of K34. It is a peculiarity of K34 that it may have two expression forms, one with a long 72-residue head group. Recent proteomic work on wool follicles has revealed unique peptides detected in the 16 residue extension of K34, suggesting

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Fig. 4 K31 localisation in early wool cortex of follicle bulb. A. Immunofluorescent micrograph of semi-thin plastic section (500 nm thick) stained with labelled antibody against K31. Micrograph has been inverted for clarity (original in inset). B. Antibody signal brightened and superimposed over Nomarski image of a follicle to illustrate location. C. A diffuse pattern of antibody label within cells of low Zone B changes to a pattern of intense elongated structures. These elongated structures have a similar morphology to that expected of tactoids and macrofibrils. D. The morphology and size of labelled structures differs between orthocortex and paracortex cells. DP, dermal papilla; GM, germinative cells; He, Henle’s layer hardens.
that the larger head form is expressed within developing fibres\textsuperscript{15}. It is notable also from Fig. 2 that K33/K83 expression overlaps with the expression of existing major keratins (K31, K85 in particular). This new potential combination of K33 and K83 is in addition to, rather than a replacement for, K31/K85 expression. This may imply that once an organised oriented mesophase has been developed the rate of filament formation can be accelerated, with K33/K85 filaments adding to existing mesophase zones. There is some observational evidence that at about the point where K33/ K83 expression commences the number of macrofibrils ceases to increase, but they get bigger\textsuperscript{25}.

7. Phase separation behaviour and cell classification

Our earlier work\textsuperscript{1} discussed how, based on the results and calculations provided by Flory and his co-workers\textsuperscript{6,7,26}, phase separation of differing rod length mixtures can produce macrofibrils with filament arrangements found in paracortex cells and in orthocortex cells. In describing cell types, based on their macrofibril arrangement, we adopt the cell classification system first clearly defined for wool by Whiteley and Kaplin\textsuperscript{27}. In essence, paracortical cells have large fused macrofibrils in which the IFs are packed roughly parallel on a basal net that is approximately hexagonal at short range. In orthocortex, the macrofibrils are smaller in lateral extent, more discrete, with the IFs clearly twisted about the longitudinal axis, the twist increasing with radial distance from the axis. A less common cell type, mesocortex, exhibits much more precise hexagonal packing of the parallel IFs. It should be noted that clear demarcations between the cell types are only possible in certain fibres such as merino wool, and most animal fibres, including human hair, tend to exhibit a continuous gradation in cell character with a tendency for orthocortex, expressed at various levels of twist intensity, to be the prevalent form. Much fuller descriptions are available elsewhere\textsuperscript{1,28}.

Here we discuss the effect of the total concentration of macromolecules in the cytoplasm on phase separation behaviour. If trigger events leading to filament formation are delayed, the concentration of anisometric ULFs in the cytoplasm will rise. This will reduce the length and concentration of ULF polymers (short IFs) at which phase separation is initiated\textsuperscript{26}. It is also true that the mesophase is more heterodisperse than the isotropic phase; typically the mesophase contains virtually all the longer filaments but also many short ones, including probably ULFs (this facilitates the formation of small tactoids, rather than perhaps spherulitic textures as found in virus mesophases). This leads to an explanation for the differences in cortex cells.

Ortho-type cells contain macrofibrils produced by delayed polymerisation, and thus derived from tactoids formed from short filaments, with close lateral approaches. At such close approaches, inter-filament chiral twisting forces develop a double-twisted texture with the structure of a blue-phase cylinder\textsuperscript{1}. Straley\textsuperscript{29} has given a formula showing the energy required to twist nematic rods about their mutual normal; this formula has also been applied to chiral rods. The twist elastic constant is proportional to $L'$ (where $L$ is the mesogen length), and to the square of the rod number density, so observable twists will only occur for short mesogens. The effects of chiral forces are thus only visible in cells with delayed polymerisation events, with high ULF concentrations. This is the origin of double-twist structures in orthocortex cells.

Data are required to establish beyond doubt that such twisted structures are the result of chiral forces and not the result of the relaxation of bending energy into twisting energy\textsuperscript{30} within tactoids (which would lead to a racemic mixture of tactoids). We are currently investigating this point.

We suggest that when double-twist cylinders are generated there is a powerful size-selection mechanism in operation. As short filaments assemble with local double-twists, longer filaments simply will not fit into the structure, either from the point of view of lateral separation, or the overall twist energy of the mesogen. Twist energy can be reduced by incorporating short rods (thus relaxing twist energy at the rod ends). Thus, in an orthocortex cell, each developing macrofibril template will be formed of filaments of roughly equivalent size; the longer the class of filaments selected the lower will be the twist intensity. Ultimately, all filaments might grow until the same filament surface energy, dependent on bending and twisting, is attained, and this might be expected to result in much the same surface tilt angle in all templates. But this depends on a plentiful local supply of free filaments of all lengths, which is probably not the case, so some tilt angle variation is only to be expected.

Explanations for the meso- and orthocortex remain much as given before\textsuperscript{1}, except for the related realisation that size selection effects will also occur when longer quasi-parallel filaments are involved, leading to an annealing process producing local regions of equivalent length filaments, in an accurate hexagonal network. This is an additional factor favouring mesocortex cell formation. In paracortex cells, resulting from earlier initiation of filament formation, filaments may be so long that such disentanglement by annealing is not possible. The observed macrofibril structure is then essentially an apposition of unmerged or partially merged tactoids, compatible with what is observed in cross section.

8. KAPs and the Mesophase Model

The key events discussed above that initiate macrofibril template formation are largely complete, or well advanced, before keratin associated proteins (KAPs) are expressed in the cortex\textsuperscript{31}. Therefore we confine our remarks here only to the way in which the keratin-based template may affect KAP incorporation. The mesophase model has the benefit that the early-stage filaments are not in physical contact, making it thus more credible that KAPs can infiltrate the structure. Another model of filament formation\textsuperscript{32}
depends on exothermic interactions between the filaments which must then be somehow undone to allow KAP entry. On the other hand, the thermodynamic model of mesophase formation that we employ depends upon the mesophase forming initially with rigorous exclusion of non-rod components. However, as small tactoids coalesce there will be a re-equilibration of rod lengths as the tactoid contents approach the equilibrium distribution. Such an ongoing chain extension is necessary to comply with X-ray diffraction data.[33]. The mesophase must then expand, because the equilibrium separation distance will increase. If the mesophase expands, some or all of the cytoplasm must be sucked into the mesophase interstices.

This offers some hope for an explanation of KAP introduction.

KAP research is currently undergoing an overdue renaissance, with interesting results appearing demonstrating specific links between KAP molecules and filaments[34,35]. The mesophase model has certain logical implications for the KAP proteins, which offer fascinating topics for future research.

9. Summary

The mesophase model can be brought into broad overall agreement with the principal spatio-temporal patterns of keratin expression, but there are doubts about the specific triggers that initiate filament formation. There is nothing in the expression pattern that specifically contradicts the mesophase model.

The most resounding success of the mesophase model, in our view, is the way it predicts the variations in cortical cell classification.

Problems remain as to how matrix proteins are incorporated but on the whole the mesophase model has potentially more to offer in that regard than any other current model. Identifying the function of KAPs within a mesophase concept is a fascinating future challenge.

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