Connective Tissue Polarity Unraveled by a Markov-Chain Mechanism of Collagen Fibril Segment Self-Assembly

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ABSTRACT The well-established occurrence of pyroelectricity (Lang, 1966) in tissues of living organisms has found a first explanation by a Markov-chain mechanism taking place during collagen fibril self-assembly in extracytoplasmic channels. Recently reported biochemical findings on the longitudinal fusion reactivity of small fibril segments (which undergo C-, N- and C-, C- but not N-, N-terminal fusions; see Graham et al., 2000; Kadler et al., 1996) may provide a mechanism by which a difference in the fusion probabilities P_{CC}, P_{NN} drives the self-assembly into partial macroscopic polar order. In principle, a Markov-chain growth process can lower the noncentrosymmetric $\infty 2$ symmetry describing dielectric properties of a growing limb (as managed by fibroblasts) into the polar ∞ group. It is proposed that macroscopically polar properties enter the biological world by a stochastic mechanism of unidirectional growth. Polarity formation in organisms shows similarity to effects reported for molecular crystals (Hulliger et al., 2002).

INTRODUCTION

Physical and biological: theory of polarity

Piezoelectricity (Shamos and Lavine, 1967) and pyroelectricity (Lang, 1966) are fundamental properties of connective tissues reported for bones, teeth, and tendons of vertebrates (Athenstaedt, 1970), the integument of vertebrates and arthropods (Athenstaedt et al., 1982; Simhony and Athenstaedt, 1980), nerve structures (Baas et al., 1988; Athenstaedt, 1984), and also for plants (Simhony and Athenstaedt, 1980). Tissue polarity is functional as organisms have sensory receptors to detect mechanical and thermal stimuli from the environment. Examples are the piezoelectric auditory membrane of the ear of *locusta migratoria* (Athenstaedt, 1985) and the infrared-sensitive (Bullock and Cowles, 1952; Newman and Hartline, 1982) organ of *Crotalinae* and *Boidae*. It is generally accepted that collagen-type fibrils arranged into parallel order by regulatory functions of fibroblasts (Trelstad, 1977; Athenstaedt, 1970) are responsible for these dielectric properties of natural tissues. However, no mechanism unraveling the occurrence of pyroelectricity is known for the morphogenesis of the extracellular matrix. A Markov-chain mechanism (Hulliger et al., 2002, 2001) and recent results on the chemical recognition driving the longitudinal self-assembly of collagen fibril segments (Graham et al., 2000; Kadler et al., 1996) will be used to explain partial macroscopic polar order of fibrils in connective tissues by a stochastic process of fibril elongation.

Markov-chains are known to model chemical and physical processes (Gardiner, 1997) represented by consecutive events $E_0 \rightarrow E_1 \rightarrow E_2 \rightarrow, \ldots, \rightarrow E_q$. A simple Markovian chain results if a transition matrix P, Eq. 5, involving constant probabilities P_{ii} and P_{ij} promotes an initial vectorial state S_0 stepwise into a final one S_q by Eqs. 1–4:

$$S_1 = P\,S_0, \tag{1}$$

$$S_2 = P\,S_1, \tag{2}$$

$$S_3 = P\,S_2, \tag{3}$$

$$\ldots$$

$$S_q = P^q\,S_0, \tag{4}$$

where $q = 1, 2, \ldots, \infty$.

Markov-type polarity formation was first discussed and experimentally demonstrated for channel-type inclusion crystals where dipolar guest molecules at the level of a seed (S_0) are arranged in a centrosymmetric packing but undergo polar alignment as growth proceeds ($E_0 \rightarrow E_1 \rightarrow, \ldots$) (Hulliger et al., 1997; Roth et al., 1998). A generalization to molecular crystals built up from dipolar but achiral molecules was recently presented (Hulliger et al., 2002).

Applied to macroscopic polarity formation resulting from the alignment of polar building blocks such as short collagen fibril segments, the vector S may be represented by the molar fractions X_C and X_N describing the proportions of fibrils pointing in the direction of growth (X_C: C-terminus; X_N: N-terminus). The values X_C and X_N depend on a growth variable q accounting for consecutive steps E_i of the longitudinal self-assembly into large fibrils. The resulting average polarization $\langle P \rangle$ of tissues may be defined by $\langle P \rangle$ proportional to $\mu_{fibril} X_{net}$, where $X_{net} \equiv X_N - X_C$ is the net fraction of aligned dipoles (μ_{fibril}). Denoted in matrix form, Eq. 4 transforms into Eq. 5:

$$\begin{bmatrix} X_C(q) \\ X_N(q) \end{bmatrix} = \begin{bmatrix} P_{CN} & P_{NN} \\ P_{CC} & P_{NC} \end{bmatrix}^q \begin{bmatrix} 0.5 \\ 0.5 \end{bmatrix}, \tag{5}$$

where P_{CN} is the probability for fusing C- and N-termini, P_{NN} that for coupling of N-termini, etc. Note that $P_{CN} + P_{CC} = 1$ and $P_{NN} + P_{NC} = 1$. The asymptotic limit for net

Submitted June 27, 2002, and accepted for publication December 3, 2002.

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0006-3495/03/06/3501/07 $2.00

polarity is $X_{net}(\infty) = (1 - \varepsilon)/(1 + \varepsilon)$, where $\varepsilon = P_{NN}/P_{CC}$. In terms of probabilities, there is only one parameter of free choice which determines $X_{net}(\infty)$. The sign of X_{net} is related to the difference of $P_{CC} - P_{NN} = \Delta P$; for example, if $\Delta P > 0$, N-termini are preferably oriented in the direction of growth.

Markovian chains feature an interesting property: the final state E_q ($q \rightarrow \infty$) does not depend on the initial state E_0. Applied to the present topic, initial values of $X_C(q = 0) = X_N(q = 0) = 0.5$, i.e., an equal distribution of short segments presenting either their C- or N-terminus in the direction of growth (distal) can undergo a development into a macroscopic state showing $|X_C - X_N| \neq 0$. This means that as a result of longitudinal growth of tissues, polarity may evolve.

As a graphical illustration of polarity evolution (Fig. 1) we have set $P_{CC} = 0.5$ and $P_{NN} = 0$. In this case, significant net polarity ($X_{net} = 0.5$) is obtained already for $q = 1$. In view of using published (Graham et al., 2000; Kadler et al., 1996) biochemical data on self-assembly reactions, Fig. 2 shows a situation where both P_{CC} and P_{NN} are realistically small. The function $X_{net}(q)$ implies that tissues may show a spatially inhomogeneous distribution of polar properties.

Different from crystals mentioned previously, collagen fibrils show an upper limit of length (about hundreds of μm to mm; Craig et al., 1989; Graham et al., 2000). As curves in Fig. 2 exceed saturation at large q, an assembly of short fibrils may not reach the asymptotic limit for X_{net}. Each new growing fibril represents a restart for a single Markov-chain.

For a discussion of tensorial properties of nonsingle crystalline composite materials such as tissues, macroscopic states are described by continuous point groups (Shubnikov, 1946). In the case of collagen based materials (a chiral and polar building block) there are two groups for representation: i), $\infty 2$ and ii), ∞. An $\infty 2$ symmetry may be found for a nematic liquid crystal composed of chiral molecules. $\infty 2$ shows piezoelectric properties but no longitudinal effect and no pyroelectricity. In ∞ we may find nematic liquid crystals made of chiral and polar molecules. Thus, the lowering of symmetry from $\infty 2$ to ∞ allows for a longitudinal piezoelectric and a pyroelectric effect.

Whatever mechanism may be found responsible to effect longitudinal (Fukada and Yasuda, 1964) piezoelectric (d_{333}), second order nonlinear optic (Freund et al., 1986) (χ_{333}), and pyroelectric (Athenstaedt, 1970; Lang, 1966) (p_3) properties in biological tissues grown from collagen, the symmetry elements describing the packing of active components in the composite material need to be those of the polar continuous group ∞. To observe a transversal piezoelectric and second order nonlinear optic effect, transformation according to the noncentrosymmetric group $\infty 2$ is sufficient. As a matter of packing we can easily understand the occurrence of i), trans-

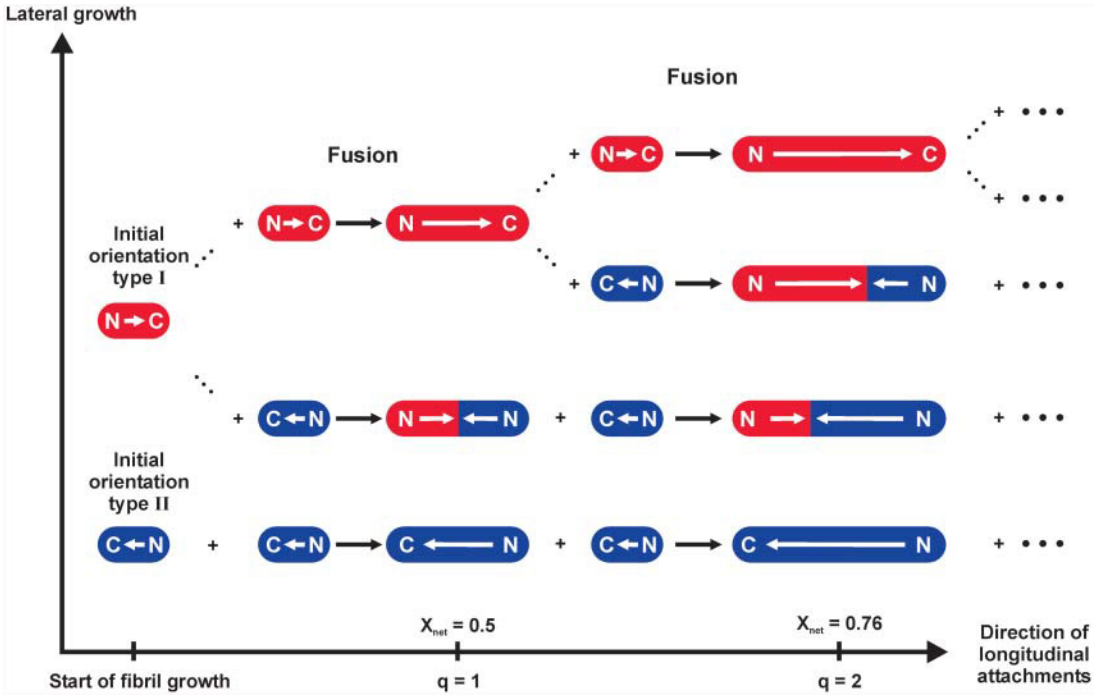

FIGURE 1 Schematic representation of polarity formation by chainlike fusion of fibril segments (*red* and *blue blocks*; *arrows* indicate the polarization within uni- or bipolar small and large fibrils) at sites I and II. For illustration $P_{CC} \approx P_{CN} = 0.5$ was assumed. $P_{CC} > P_{NN}$ follows from reactions 2 and 3 in Fig. 5. Because of $P_{CC} > P_{NN}$ net polarization $\langle P \rangle$ ($\langle P \rangle$ being proportional to $X_{net}(q) = X_N(q) - X_C(q)$) is oriented in the direction of growth. According to data reported by Kadler et al., 1996, and Graham et al., 2000, it is likely to have $P_{CC} \ll P_{CN}$. Consequently, a larger number than 1 or 2 of attachment steps q are needed to obtain some polarity $\langle P \rangle$.

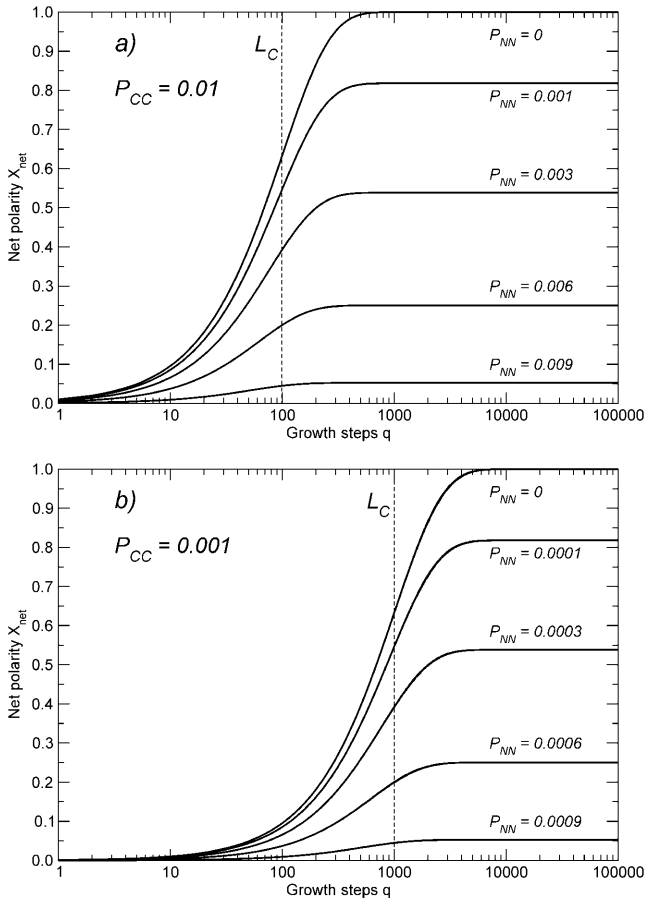

FIGURE 2 Net polarity $X_{\text{net}} \equiv X_{\text{N}} - X_{\text{C}}$ as a function of growth steps q (fibril length: length of building blocks $\times$ q)). Input parameters are the probabilities P_{CC} and P_{NN} of Eq. 5. Asymptotic limits show that $X_{\text{net}}(\infty)$ can be described by a single parameter $P_{\text{NN}}/P_{\text{CC}}$. Vertical broken lines: estimated characteristic lengths L_{C} for unipolar fibrils. For calculating curves in *a* and *b*, we have chosen values for P_{CC} and P_{NN} discussed in the text.

versal piezoelectricity and ii), optical nonlinearity of tissues showing preferably a parallel alignment of collagen fibrils. However, understanding the existence of pyroelectricity will need further arguments for lowering the symmetry.

In vivo self-assembly of collagen into ∞ requires thus a mechanism for a vectorial type of alignment of building blocks, whereas in $\infty 2$ blocks will just have to align into parallel arrays, featuring an equal number of dipoles pointing in either direction of growth. Whereas a high degree of parallel alignment ($\infty 2$) of collagen fibrils is known for tendons, bones, and other tissues (Weiner and Wagner, 1998), in collagen rich tissues (tendon) net vectorial alignment (∞) is in the order of only a few percent (nonlinear optics; Freund et al., 1986). Evidence for an inhomogeneity of the second harmonic response was reported as well (Kim et al., 1999). However, no systematic analysis with respect to the longitudinal variation of polarity is available so far. On average $\sim$53% of the fibrils in chicken tendon were found oriented with the N-terminus in the direction to the end of the limb, and 47% with the N-terminus in the opposite direction (electron microscopy; Trelstad et al., 1983; Trelstad and Birk, 1984). As found by pyroelectric measurements (Athenstaedt, 1970), in tendons and bones, on average the positive pole (N-terminus) of polarization is pointing in the direction of biological growth (distal). Given the fact that some tissues develop in both directions of the axial morphology of a limb, bipolar bones are typical for the skeleton of vertebrates (Athenstaedt, 1970).

The embryonic morphogenesis of prolate shaped tissue is regulated by mesenchymal cells that are flattened and inherently polar (Trelstad and Hayashi, 1979). Fibroblasts in tendon show a high degree (up to $\sim$50%–70%) of vectorial alignment parallel to the long axis of a growing limb (Trelstad and Birk, 1984; Holmes and Trelstad, 1977). An obvious correlation between patterns of aligned cells and tissue polarity was recognized earlier (Trelstad, 1977; Trelstad et al., 1983; Trelstad and Birk, 1984), however, without giving a mechanistic explanation for the existence of pyroelectricity. Mechanical forces (Stopak et al., 1985; Harris et al., 1981) exerted onto fibrils being self-assembled within extracytoplasmic channels promote a vectorial discharge of fibrils mainly in the distal direction (Trelstad, 1982). A summary on cellular and extracellular processes leading to fibril formation is provided by Fig. 3 (after Trelstad and Hayashi, 1979).

As a matter of present knowledge, fibroblasts primarily arrive to structure an undifferentiated blob of mesenchyme into a limb featuring $\infty 2$ symmetry allowing for some third rank tensorial properties of the composite material.

The principal aim of the present paper thus is to provide more or less qualitative arguments for a growth mech-

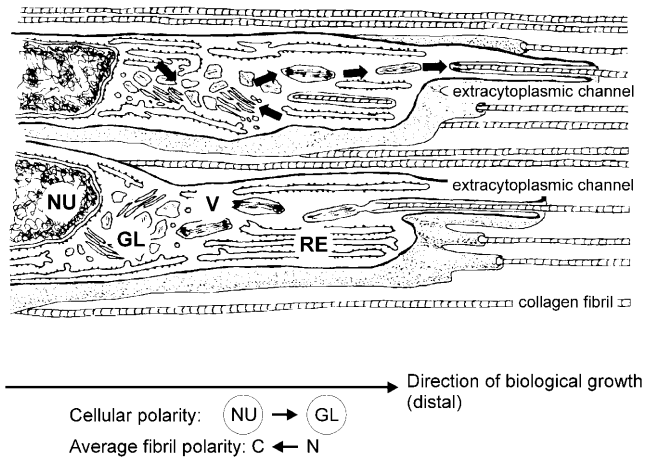

FIGURE 3 Diagram illustrating the production of fibrils by fibroblasts (redrawn after Trelstad and Hayashi, 1979). GL, Golgi apparatus; NU, nucleus; RE, endoplasmic recticulum; V, vacuoles. Bold arrows in the upper part: translocation of procollagen across the cell. The cellular polarity (vector pointing from *NU* to *GL*) is oriented in the longitudinal direction of growth. The polarity of fibrils (plus end) as found by electron microscopy and pyroelectric measurements is preferably featuring N-termini in the distal direction.

anism lowering the symmetry from ∞2 (nonpolar) to ∞ (polar).

On the origin of polarity in tendon

Tracing back the origin of tissue polarity necessitates an analysis (Fig. 4) of the microscopic (p) and macroscopic ($\langle P \rangle$) polarization along the entire process of growth, starting by collagen peptide chain formation and ending by fibrils cross-linked at final positions: molecular polarity p of procollagen results from translation in the endoplasmic recticulum (Trelstad, 1982). Transported by secretory granules to the Golgi apparatus, procollagen self-assembles into SLS-type aggregates (up to three molecules long (0.845 μm); Silver et al., 1979; Gross and Bruns, 1984). Finally these precursors are secreted into extracytoplasmic channels for further self-assembly, after cutting of C- and N-propeptides. Polar intracellular aggregates are stabilized by electrostatic interactions (Silver, 1982). It was proposed that small polar collagen fibril segments are the genuine building blocks for subsequent growth of long fibrils in channels (Birk et al., 1989, 1997). Because of a rotational diffusion coefficient (Silver, 1982) for 4D-staggered trimers of collagen of $\sim 40\ s^{-1}$ and intracellular processing and transport times (autoradiographic analysis (Weinstock and Leblond, 1974)) in the order of minutes to hours, we are allowed to conclude that $\langle P \rangle_{\text{intracellular}}$ is 0. These findings are supported by studies (Polishchuk et al., 2000) on the intracellular traffic between the Golgi apparatus and the plasma membrane (correlative light-microscopy using the green fluorescent protein technology).

Fibril segments subjected to further processing arrive in extracytoplasmic channels by random vectorial orientation, providing thus a stochastic input for a Markov-chain process of longitudinal fibril assembly. Recent electron microscopy studies have established fusion reactions summarized in Fig. 5 (Kadler et al., 1996; Graham et al., 2000): there exist unipolar and bipolar fibrils in early tendon, notably fusion of N-termini was not observed. These reactions provide an available but preliminary base for a mechanism that may effect a difference in the error probabilities P_{CC} and P_{NN} used in Eq. 5.

In terms of a Markov-chain model (Hulliger et al., 2002, 2001) for explaining polarity formation we denote two unidirectional channels, termed sites I and II (Figs. 1 and 4), being distributed across a limb. We anticipate that therein fibril polarity pointing in the distal (I) or proximal (II) direction is developing (see also Fig. 3). Given a delivery of building blocks featuring a random orientation when arriving at sites I and II, the system fulfils conditions of a Markov-chain description These sites are equally probable for

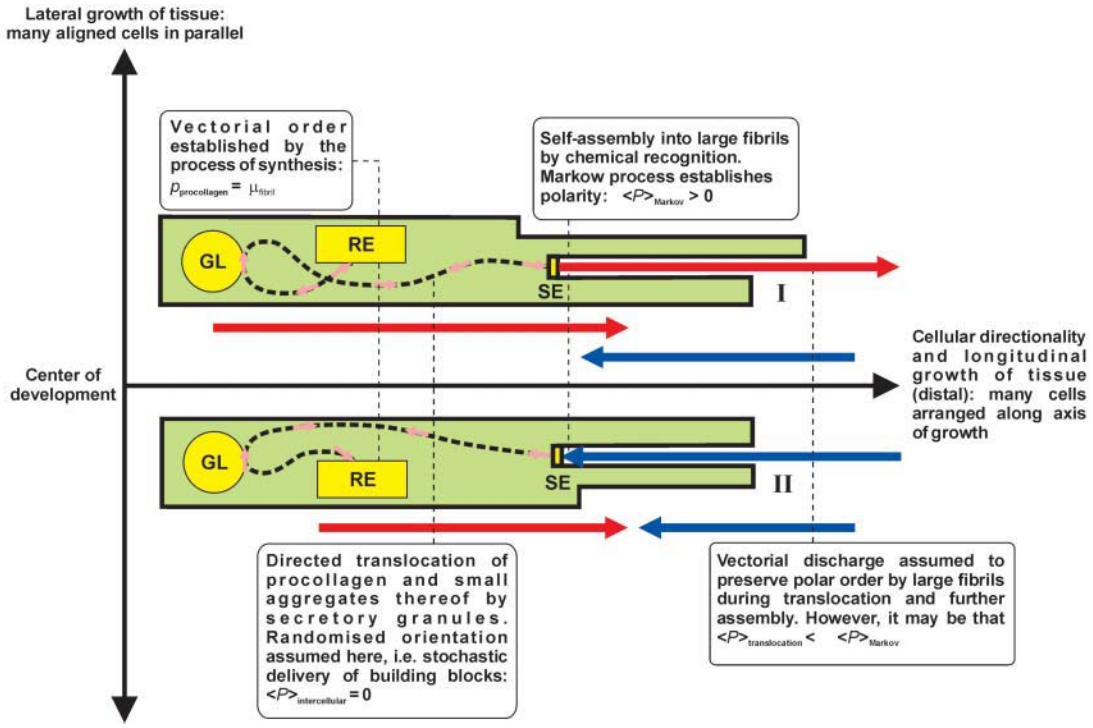

FIGURE 4 Mechanistic analysis of polarity formation during the process of tissue growth by regulatory functions of cells (*green part*). Cells and produced fibrils are interwoven. For related diagrams in biological work, see Trelstad et al., 1984, 1977, and Fig. 3. RE, endoplasmic recticulum; GL, Golgi apparatus; SE, secretion into extracytoplasmic channels. I and II: two representative extractytoplasmic channels where assembly into large fibrils is assumed to occur (Trelstad and Hayashi, 1979; Birk et al., 1989, 1997). Many such sites I and II are distributed over a growing limb. $p_{procollagen}$: polarization of the molecule; $\langle P \rangle$: macroscopic polarization. Red and blue arrows: grown fibrils. As a result of the Markov process (here $P_{CC} > P_{NN}$; see reactions 2 and 3 in Fig. 5) the sum of the red vectors is larger than that of the blue ones. Small pink arrows: intracellular translocation of procollagen and aggregates thereof.

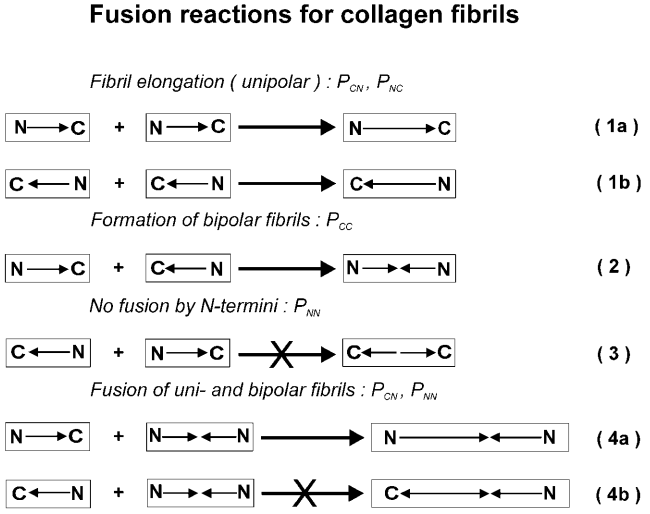

FIGURE 5 Reactions should be read in the way that fibrils are growing in extracytoplasmic channels. Building blocks (*mostly to the left*) are small segments (precursor aggregates). Blocks to the right represent growing fibrils of increasing length. Bipolar fibrils are not considered as precursors here. (After data by Kadler et al., 1996; Graham et al., 2000.)

initiating fibril polarity by fusion of segments (*red* and *blue arrows* in Fig. 4). There is no assumption made here on any possible mechanism giving rise to $X_C(q=0) \neq X_N(q=0)$ for sites I and II, before self-assembly becomes effective.

Translation of present information on reactions 1–4 in Fig. 5 into fusion probalities P_{ii} may imply: $P_{CC} > 0$, $P_{NN} = 0$. As fusion by N-termini was not observed for tendon (embryonic chick metatarsal leg; Graham et al., 2000), we are allowed to conclude that $P_{CC} \neq P_{NN}$. Given a difference in P_{CC} and P_{NN}, a basic argument for polarity formation in tendon is found.

Taking into account that i), fibrils can reach a length of several hundreds of μm (Craig et al., 1989; Graham et al., 2000), ii), growth occurs from short blocks (Birk et al., 1989, 1997) and, iii), bipolar fibrils feature normally a single inversion of their polarization (Kadler et al., 1996; Graham et al., 2000), it is likely to have $P_{CN} \gg P_{CC}$. A rough estimate for P_{CC} may be obtained from the characteristic length L_C representing uniform chains (Hulliger et al., 2001): $P_{CC} = 1/L_C$; assuming building blocks (SLS-type) of ≈ 1 μm or less, and an unperturbed average fibril length up to hundreds of μm, L_C may be of the order of 100–1000, therefore, $P_{CC} \approx 0.001$–0.01. Entering Fig. 2 by these values yields a proportion of polar alignment that is much larger than reported by two independent experimental studies: 1), electron microscopy, where $X_{net}(q) \approx 0.06$ (Trelstad et al., 1983; Trelstad and Birk, 1984), and 2), nonlinear optical techniques where a ''few percent'' of the collagen material in tendon shows polar order (Freund et al., 1986). However, setting $P_{NN} = 0$ is an unrealistically strong condition. Because of this, it is more likely to have $P_{CC} > P_{NN}$, both being small. For such conditions much lower X_{net} values below or at $q = L_C$ are possible.

Another factor reducing X_{net} in real tendon is the distribution of lengths for fibrils. In the case when most fibrils are shorter than L_C, average polarity may become as small as that found experimentally.

Concerning the sign of X_{net}, a situation providing P_{CC} larger P_{NN} would imply that N-termini are preferably pointing in the direction of growth (Fig. 1) as found experimentally. However, from Fig. 3 it is likely to conclude that fibrils are elongated from their terminus close to the inner end (proximal) of the extracytoplasmic channels. In this case C-termini would come to point in the direction of growth.

A refined prediction of X_{net} including its sign would thus require more experimental details than presently available for processes retrieved by Fig. 3 and on reactions summarized in Fig. 5.

DISCUSSION

As presented, we have not addressed the influence of lateral interactions during fibril aggregation in extracytoplasmic channels. From a general discussion of Markov-type polarity formation in crystals (Hulliger et al., 2002) we know that lateral interaction between building blocks can operate in favor or against the unipolar alignment of polar building blocks. It may well be that lateral interactions strongly favor polar fibril formation (Silver, 1982). However, production of nothing but unipolar fibrils (on average of the same length, see below) at all independently producing sites I and II would yield zero net polarity (Fig. 4). At this point we recognize a situation analogous to polarity formation in topologically centrosymmetric molecular crystals (Hulliger, 1998; Hulliger et al., 2001, 2002): a pyroelectric symmetry is found in growth sectors of crystals if corresponding probabilities driving orientational disorder at growing crystal faces are different. In the present case we just need to have $P_{CC} \neq P_{NN}$, where present knowledge on reactions 2 and 3 of Fig. 5 implies that $P_{CC} > P_{NN}$.

So far, elements of the transition matrix in Eq. 5 were discussed without any recourse on kinetic effects. It might well be that the kinetics of reactions 1 *a* differ from 1 *b*. This would result in a production of unipolar fibrils of a different length with respect to sites I and II. Independent of the effect of $P_{CC} \neq P_{NN}$ as anticipated by reactions in Fig. 5, kinetic factors may contribute to polarity formation. However, present knowledge on reactions 1–4 does not provide enough data for denoting a significant kinetic driving force entering a Markov-chain description. Instead, we can argue for having found a possible driving force that allows an assembly of fibrils to become polar. It is important to notice that fibroblasts must preferably show a release of fibrils in either the distal or the proximal direction (Fig. 3). However, this asymmetry is not being described by the present Markov model. As we have shown, a directional asymmetry in the

production of fibrils by fibroblasts would not be sufficient to explain the occurrence of macroscopic polar ordering.

SUMMARY AND CONCLUSIONS

More than 35 years after its discovery (Lang, 1966; Fukada and Yasuda, 1964) a Markov growth model seems to unravel the origin of a widespread phenomenon found for growing organisms: we propose that polarity (∞) of tissues is entering the biological world by a stochastic mechanism, breaking the $\infty 2$ symmetry as managed by the morphogenesis of fibroblasts.

Using recent biochemical data on the self-assembly of collagen fibrils for an estimate of probabilities, the present theory agrees with the following experimental findings: i), Tissues can become pyroelectric; and ii), growth in both directions leads to a bipolar limb or bone. With respect to the proportion of polar alignment, the Markov model using present biochemical data predicts larger values for X_{net} than reported by electron microscopy and second harmonic generation studies. However, assuming reasonable probabilities for the self-assembly process can lead to a prediction of polar order of a few percent as observed. More quantitative data on the extent and spatial distribution of polarity in tissues including knowledge on the size distribution of fibrils would be necessary for a refined quantitative prediction of polar order; iii), With respect to the sign of X_{net} the Markov description by Eq. 5 using $P_{\text{CC}} > P_{\text{NN}}$ predicts that an excess of N-termini are pointing in the direction of the attachment process. In case fibrils undergo enlargement mainly from the proximal side within channels, the experimental result (N-termini in distal direction) is not being reproduced if using $P_{\text{CC}} > P_{\text{NN}}$.

I thank T. Wuest for calculating Fig. 2.

This work was supported by the Swiss National Science Foundation, NFP 47, Functional Supramolecular Materials, No. 4047-057476/1.

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