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Sequential Assembly of Collagen Revealed by Atomic Force Microscopy

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ABSTRACT Most polymers which comprise biological filaments assemble by two mechanisms: nucleation and elongation or a sequential, stepwise process involving a hierarchy of intermediate species. We report the application of atomic force microscopy (AFM) to the study of the early events in the sequential or stepwise mode of assembly of a macromolecular filament. Collagen monomers were assembled in vitro and the early structural intermediates of the assembly process were examined by AFM and correlated with turbidimetric alterations in the assembly mixture. The assembly of collagen involved a sequence of distinctive filamentous species which increased in both diameter and length over the time course of assembly. The first discrete population of collagen oligomers were 1–2 nm in diameter (300–500 nm in length); at later time points, filaments ~2–6 nm in diameter (>10 μm in length) many with a conspicuous ~67-nm axial period were observed. Occasional mature collagen fibrils with a ~67-nm axial repeat were found late in the course of assembly. Our results are consistent with initial end-to-end axial association of monomers to form oligomers followed by lateral association into higher-order filaments. On this basis, there appears to be at least two distinctive types of structural interactions (axial and lateral) which are operative at different levels in the assembly hierarchy of collagen.

INTRODUCTION

The function of many biological macromolecular complexes is closely related to their higher-order structure that is produced through poorly understood self-assembly processes. Among the most complex self-assembling biological systems are the membranous organelles, nuclear pore complex, viruses, and the filaments of the cytoskeleton and extracellular matrix. In general, the polymers which comprise various biological filaments assemble by two well characterized mechanisms: 1) nucleation and elongation or 2) a stepwise or sequential process involving a hierarchy of intermediate species. The assembly of tubulin is the paradigm nucleation and elongation process which explains the formation, growth, and dynamic instability of microtubules (Fygenson et al., 1994). However, several polymeric structures such as the intermediate filaments and various matrix-associated macromolecules (e.g., fibrillin and collagen) assemble by the controlled stepwise aggregation of oligomers (e.g., Aebi et al., 1983). One of the most important characterizing features of the hierarchical assembly of protein oligomers is the formation of transient but highly stable structural intermediates that can be studied using ultrastructural methods.

Collagen molecules are the main structural macromolecule composing fibrils in various connective tissues responsible for tensile strength. Due to theoretical interest in collagen assembly as well as the biological importance of collagen and its implication in inherited diseases of connective tissues, collagen fibrillogenesis has been studied for over 40 years by a wide variety of techniques, including turbidimetry (MacBeath et al., 1993), dynamic light scattering (Fletcher, 1976; Silver et al., 1979), electric birefringence (Bernengo et al., 1983; Brokaw et al., 1985), x-ray crystallography (Eikenberry and Brodsky, 1980), electron microscopy (EM) (Gelman et al., 1979; Kobayashi et al., 1985; Ward et al., 1986), x-ray scattering (Leikin et al., 1994) and dark field microscopy (Kadler et al., 1990; Notbohm et al., 1993). Collagen assembly has also been studied from a theoretical perspective (Silver et al., 1992; Wallace, 1992). These studies have been variously interpreted to indicate that collagen fibrillogenesis is a paradigm sequential assembly process in some cases, in others, that it is a nucleation and growth process. Ultrastructural studies of collagen assembly have been greatly facilitated by the axial periodicity of collagen fibrils which is due to the staggered organization of collagen molecules.

Recently, we have applied atomic force microscopy (AFM) to study the biological filaments with axial periodicity including the paired helical filaments of Alzheimer's disease and disease-associated intermediate filaments (Pollanen et al., 1994a,b). AFM is a recently developed imaging technique that produces an image of the surface topography of a specimen by measuring the response of a tip attached to a cantilever that is raster-scanned over the sample (Binnig et al., 1986). AFM is well suited for biological application (Lal and John, 1994) since, unlike electron microscopy, minimal specimen preparation is required and very high vertical resolution (<1 Å) can be achieved. For these reasons, the axial periodicity of mature collagen fibrils has been resolved by AFM (Chernoff and Chernoff, 1992; Baselt et al., 1993; Revenko et al., 1994); the results from AFM studies agree well with axial periodicity measurements obtained by TEM and SEM.

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In this report, we use AFM to characterize the stable transient structural intermediates formed during the in vitro assembly of collagen. These studies indicate that the AFM can be used as a quantitative tool to study the products present at different stages of collagen assembly.

MATERIALS AND METHODS

Assembly of collagen in vitro

Collagen fibrils were assembled in vitro using the neutral start method of Holmes et al. (1986) (also referred to in the literature as cold start). Type I acid-soluble collagen from calf skin (Sigma, St. Louis, MO) was solubilized in 0.010 M acetic acid (pH 3.7) at 4°C to an initial concentration of ~0.4 mg/ml. The type I collagen solution was brought to a final concentration of ~0.2 mg/ml by dilution with an equal volume of phosphate buffer (0.4 M Na_2HPO_4/KH_2PO_4, 4°C) to give a final ionic strength 0.2 and pH 6.8. The phosphate-buffered collagen preparation was deposited into a cylindrical glass assembly cell and the turbidity was monitored at 632 nm over 150 min. The contents of the assembly cell were continuously stirred at a very low speed and were maintained at a constant temperature (34°C) over the time course of the experiment. Subsequent experiments performed without stirring produced similar results. Aliquots of the assembly mixture were aspirated from the cell every 6 min for the first hour, every 8 min for the second hour, and every 15 min thereafter. The assembly process in the aliquots was halted by dilution of the sample into distilled water (1:10, sample/water) and the diluted suspension was immediately deposited onto freshly cleaved mica and allowed to air dry >0.5 h before viewing in the atomic force microscope.

Atomic force microscopy

AFM images were obtained using a Nanoscope II (Digital Instruments, Santa Barbara, CA) using square pyramidal silicon nitride tips (Nanoprobes, Digital Instruments) with 0.58 N/m nominal spring constant, as previously reported (Markiewicz and Goh, 1994; Pollanen et al., 1994a,b). Gain settings were set at 2 and 3 for integral and proportional gain, respectively. Scan rates were approximately 2 Hz, with all samples imaged in both the height and force mode. Typical forces were found to be ~80 nN for operating in ambient air. The height measurements reported here were taken from height mode images. Images presented were neither processed nor filtered and represent direct output from the AFM. In Figs. 2 and 3 false color is used to represent sample height; darker areas denote depression while lighter areas indicate elevation.

Ultrastructural analysis

Each diluted aliquot obtained from the assembly cell was examined by AFM. Filaments from each aliquot were qualitatively examined from eight independent randomly selected 8000 × 8000 nm fields on the mica substrate. For quantitative analysis, a frequency distribution of the diameters of filaments at various time points during the assembly process was determined by counting and measuring at least 200 filamentous species at each time point assayed. Typically, the ~200 filaments examined would be analyzed in the eight randomly selected 8000 × 8000 nm fields. Overlapping filaments and irregular material which could represent co-depositing amorphous debris were excluded from diameter determinations. Axial periodicity was determined using the cross-section mode of the Nanoscope II. Fibril lengths were determined using the horizontal measuring feature on the Nanoscope II; in the case of curved fibrils the straight segments were measured and added together with an approximation for the curved portion (the curved section was approximated as a series of straight segments).

The uncertainty (standard deviation/mean) in measurement of fibril diameters is as follows: For the monomers and oligomers the uncertainty is 32%, for 1- to 2-nm fibrils the uncertainty is 19%, for 2- to 4-nm fibrils the uncertainty is 24%, for 4- to 6-nm fibrils the uncertainty is 10%, for 6- to 10-nm fibrils the uncertainty is 15%, and for 20- to 26-nm fibrils the uncertainty is 15%. This uncertainty is primarily due to deviations in true fibril diameter, but also includes variability in fibril deposition and flatness of local regions of the substrate; the uncertainty in these measurements due to the AFM is negligible in comparison. The range of monomer and oligomer lengths deviate by ~10% above and below the mean.

The vertical deflection of the AFM probe, is a reliable method of measuring filament diameter. However, measurement of the diameter of a filament using the width of the image is unreliable due to the distortion of apparent width caused by the tip geometry. A typical measured width for a structural intermediate of collagen with a height of 2–4 nm was ~100 nm. While AFM images can be processed to minimize the lateral distortion of the AFM probe (Markiewicz and Goh, 1994) an alternative approach is to determine the magnitude of the lateral distortion of an individual AFM probe using colloidal gold (7–11 nm diameter) co-deposited with the filaments. In these experiments, such an analysis showed that the AFM probe used to study the collagen preparations accurately determined the diameter of the colloidal gold standard as indicated by the vertical displacement of the tip but the width of the same particles was determined to be 70–100 nm. These results indicate that a major part of the lateral distortion of size by the AFM probe is due to the tip shape and underscores the importance of determining diameters of nanometer scale objects using vertical displacement of the the AFM tip.

RESULTS AND DISCUSSION

Over the time course of the in vitro assembly of collagen a progressive increase in turbidity of the assembly buffer (Fig. 1 *A*) was correlated with appearance of four filamentous species easily distinguished by AFM at approximately 20, 60, and 120 min of fibrillogenesis.

Between 0–20 min of type I collagen fibrillogenesis in vitro, filaments with a diameter of 0.7–1.5 nm were the most frequent constituents of the assembly mixture and had a length of 250–300 nm, or 500–600 nm (Figs. 1 *B* and 2, *A* and *B*). The 250- to 300-nm filaments likely corresponded to collagen monomers which have a diameter of 1.5 nm and a length of 300 nm as determined by x-ray diffraction (Hodge, 1967). A cryogenic force microscopy study of collagen monomers has been performed by Shattuck et al. (1994); their use of an electron-beam deposited tip allows excellent resolution. The imaging is performed at a temperature of 143 K, under liquid isopentane, which may help to stabilize the sample. Shattuck et al. (1994) observed the flexible nature of the molecules as well as the tendency of monomers to interact with the center of other monomers. Our

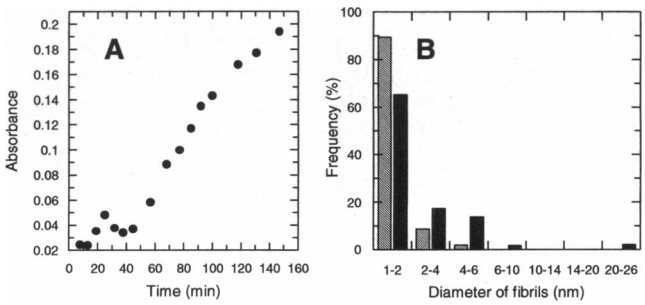

FIGURE 1 (*A*) Time course of absorbance (632 nm) of collagen assembly mixture. Assembled fibrils were imaged by AFM and quantitated after ~20 and ~60 min of in vitro assembly. (*B*) Histogram showing distributions of fibril diameters at 20 min (*stippled*) and 60 min (*solid*) of in vitro assembly.

samples, with minimal preparation under ambient conditions, are consistent with both of these observations. In Fig. 2 *A*, the Y-shaped aggregate shows the monomer-monomer interaction, and various bent shapes of monomers indicate flexibility. The 1.5-nm filaments with a length of 500–600 nm likely represent the initial products of the axial overlapping of collagen monomers to produce collagen dimers that are predicted on the basis of minimal overlap (0.4 D) to have a stable length of ~536 nm.

After 20–60 min of in vitro fibrillogenesis, the first filaments with both extended length and axial periodicity appear. These microfibrils have a diameter of 1.5–2.5 nm (Fig. 1 *B*), have a well defined axial period of ~67 nm (Figs. 2, *C* and *D*, and 4 *A*), and range widely in length. Later in the time course of assembly, long convoluted and overlapping fibrils (>10 μm) that are > 4 nm in diameter are observed (Fig. 3, *A* and *B*). The convoluted pattern of deposition on the mica substrate indicates that the fibrils are highly flexible with a shorter persistence length then mature collagen fibrils (Fig. 3, *C* and *D*) which are linear in orientation. The mature fibrils appeared longer than any of the precursor filaments (tens of μm in length), and have a clearly delineated axial period of 67 nm (Fig. 4 *C*). However, the precursor filaments were often convoluted, thus obscuring exact determination of their length.

These results indicate that different filamentous species (oligomers, microfibrils, fibrils, mature fibrils) of collagen can be observed by AFM during in vitro fibrillogenesis. Both qualitative and quantitative AFM data indicates that as collagen assembly progresses the relative abundance of the various filamentous species changes indicating a hierarchy of filaments (Fig. 1 *B*). The progressive increase in filament diameters can also be used to infer the order of the participation of the various "subfilaments" in the path to the formation of a mature collagen fibril. The progressive increase in both the diameter and length of filaments involved in collagen fibrillogenesis indicates both that axial (end-to-end) and lateral interactions of filaments are the associative forces that drive filament assembly. In the early stages of assembly, the dimerization of collagen monomers was indicated by lengthwise extension due to the minimal overlapping of two ~300-nm monomers. Such axial interactions likely mediate the early events of filament assembly in many biological filaments which assemble by the ordered association of protein oligomers. A similar example is the formation of the protofilaments (~2 nm in diameter) of intermediate filament assembly which assembly from tetrameric subunits solely by end-to-end or axial interaction (van de Klundert et al., 1993; Meng et al., 1994). In contrast, the lateral interactions of the the higher-order structural intermediates of collagen assembly lead to the formation of mature periodic filaments and is responsible for the progressive increase in filament diameters during fibrillogenesis. The physical basis of this process is poorly understood and may be related to the regular or pe-

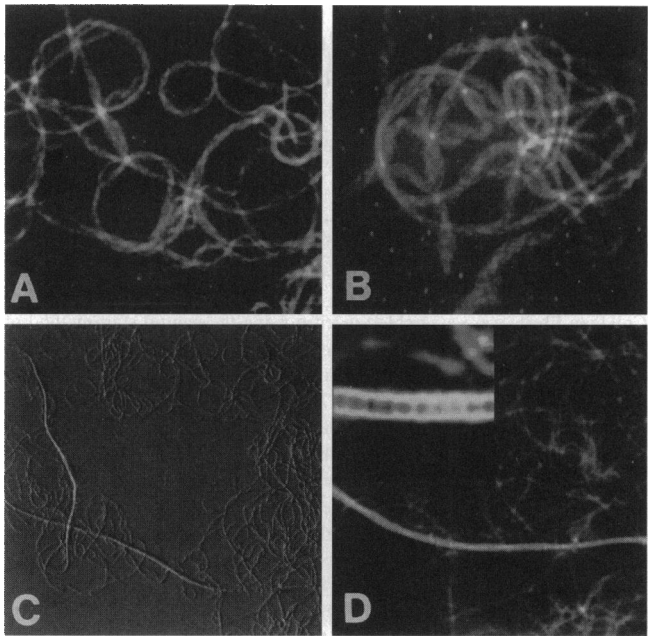

FIGURE 3 AFM micrographs showing the last two stages of fibrillogenesis. (*A*) Height mode image of larger microfibrils (diameter 4–6 nm) and oligomers (t = 40 min of assembly; horizontal scale: 3.5 × 3.5 μm). (*B*) Height mode image of large microfibrils and oligomers (horizontal scale: 3.1 × 3.1 μm). (*C*) Force mode image showing two mature fibrils (diameter 20 nm), large microfibrils and small microfibrils (t = 118 min of assembly; horizontal scale: 11.0 × 11.0 μm). (*D*) Height mode image showing one mature fibril and microfibrils from *C* (horizontal scale: 5.0 × 5.0 μm). Inset showing mature fibril from *D*; 67 nm periodicity is evident (horizontal scale: 675 × 675 nm).

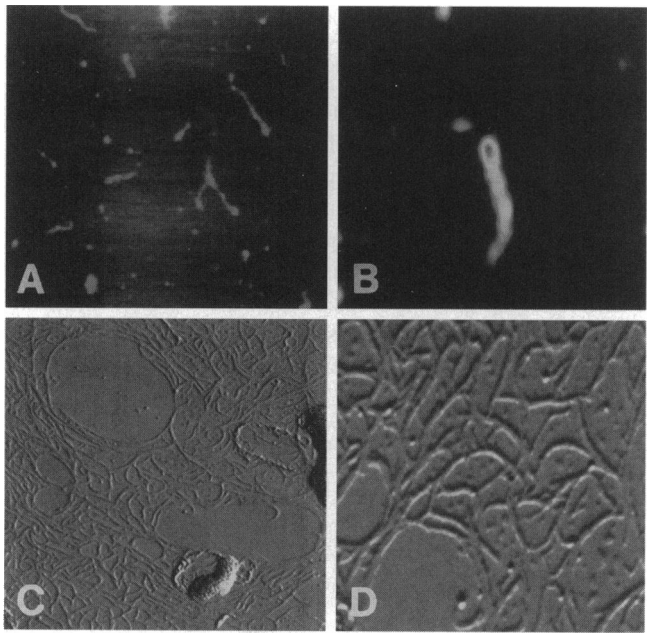

FIGURE 2 AFM micrographs showing the first two stages of fibrillogenesis. (*A*) Height mode image of starting material showing several monomers and oligomers (diameter 0.7–1.5 nm; horizontal scale: 3.0 × 3.0 μm). (*B*) Height mode image of monomer (~300 nm in length) from starting material (horizontal scale: 800 × 800 nm). (*C*) Force mode image of a network of small (diameter ~2 nm) microfibrils (t = 25 min of assembly; horizontal scale: 8.0 × 8.0 μm). (*D*) Several small microfibrils from *C*; horizontal scale: 3.0 × 3.0 μm.

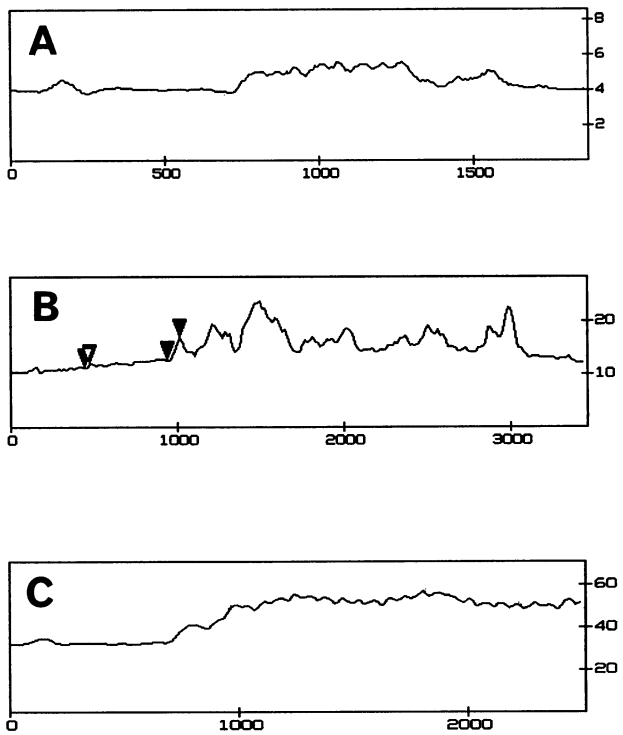

FIGURE 4 Sections through collagen fibrils; axes are vertical distance (nm) vs. horizontal distance (nm). (*A*) Section along long axis through fibrils in Fig. 2 *C* showing periodicity of ~67 nm. (*B*) Section along short axis through fibril and oligomer in Fig. 3 *B*, showing height difference (fibril 4.3 nm; oligomer 0.9 nm; height is difference in height between arrowheads). (*C*) Section along long axis through fibril in Fig. 3 *D* showing periodicity of ~67 nm. Note both microfibrils (*A*) and mature fibrils (*C*) show 67-nm periodicity.

riodic distribution of amino acid side chains on the surface of the filaments.

The mode of assembly observed in studies of collagen fibrillogenesis depends partly on the experimental conditions used as well as the particular stage of assembly studied. Recent microscopic studies of collagen assembly (Kadler et al., 1990; Holmes et al., 1992) have suggested growth by nucleation and elongation. The dark field microscopy study by Kadler et al. show mature fibrils growing from pointed tips (structural nuclei). A helical model of nucleation and propagation was proposed to explain these observations. In this model a structural nucleus composed of monomers elongates by the addition of monomers at pointed tips of the growing fibril. When this model was applied in a computer simulation it produced paraboloid tips of the same dimensions as observed experimentally (Silver et al., 1992). Since the microscopy study was performed on mature fibrils it does not exclude the possibility of stepwise growth at earlier stages of collagen assembly. Stepwise growth in the earlier stages of assembly could produce fibrils which then act as the structural nuclei for nucleation and growth.

SUMMARY

We have monitored the fibrillogenesis of type I collagen using AFM to visualize different stages of assembly. The differential distributions of fibril diameters as assembly progresses show a hierarchy indicating a four stage assembly process. Assembly shows a progression from oligomers to small microfibrils, then larger microfibrils followed by mature collagen fibrils. Assembly proceeds initially by axial overlap of monomers to form oligomers, followed by lateral interactions to form larger fibrils. Periodicity was observed and measured in small microfibrils and mature fibrils; both showed a periodicity of ~67 nm. The formation of collagen fibrils is one example of the assembly of a macromolecular complex. We have shown that the AFM is an effective quantitative and qualitative tool to study the assembly of biological filaments. Application of AFM to other self-assembling biological structures will contribute to understanding the basic principles which govern their assembly.

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