Type I Collagen Fibrillogenesis in Vitro
ADDITIONAL EVIDENCE FOR THE ASSEMBLY MECHANISM*

Frederick H. Silver
From the Department of Pathology, Shriners Burns Institute, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114

The intensity of scattered light at angles between 30° and 120° has been measured during the heat gelation lag phase of rat tail tendon collagen. As previously reported, the intensity at a scattering angle of 90° does not change during the lag phase of gelation, whereas during this period, the intensity extrapolated to zero degrees more than doubles. Based on measurement of the Rayleigh factor at low angles, it is concluded that the lag phase terminates when the molecular weight is greater than 930,000 which is consistent with the formation of a linear 4D staggered trimer as previously proposed.

Once lateral growth begins, the molecular weight continues to increase approximately linearly with time until a molecular weight of 4 × 10⁶ (5 ± 1 trimers) is reached, at which time the rate of increase of molecular weight increases significantly. It is concluded that a trimer with about 5 strands forms during the early phases of lateral growth and appears similar to the microfibrillar unit proposed based on x-ray diffraction modeling. Further growth occurs by linear and lateral addition of the trimeric units in a manner still under investigation.

Type I collagen fibrillogenesis and gelation in vitro is a multistep process which occurs spontaneously at neutral pH and physiologic temperature. Early turbidity studies (1) indicated that fibril growth occurred after a lag phase which was interpreted as the period when fibril precursors formed (2). Although a careful study by Comper and Veis (3) of the lag phase by viscometry, sedimentation equilibrium, and light scattering failed to reveal the formation of aggregates, electron microscopic observations (4) and laser light scattering studies (5) indicate that aggregation does indeed occur during this period. Based on measurement of the translational diffusion coefficient and the intensity of scattered light at an angle of 90°, Silver et al. (5) proposed that collagen single molecules form linear 4D staggered dimers and trimers before lateral aggregation occurs. Studies by other workers (6, 7) suggest that very long thin linear aggregates form during the lag phase which laterally aggregate during the growth or secondary phase of turbidity-time curves.

In order to further characterize the nature of aggregates formed during turbidity lag and growth phases, multangle intensity measurements as well as molecular weight determinations have been made. The results of these studies indicate that the turbidity lag phase is characterized by a single growth mechanism, whereas the growth phase is composed of at least 2 mechanisms.

MATERIALS AND METHODS

Rat Tail Tendon Collagen—Acid-soluble rat tail tendon collagen was prepared and characterized as previously reported (8) and was neutralized at 4 °C by adding to it an equal volume of potassium phosphate (K₂HPO₄/KH₂PO₄) buffer, pH 7.6, ionic strength 0.4.

Laser Light Scattering—Molecular weights and translational diffusion coefficients were determined as previously reported (8) using a Chromatix KMX-6 light-scattering device and a Langley-Ford correlator. All diffusion coefficients were obtained by making a least squares fit of the correlation function using a PDP 11/03 minicomputer interfaced with the correlator.

Aliquots of samples to be gelled were characterized using the Brice Phoenix light-scattering device to measure the particle-scattering factor and the Chromatix KMX-6 for diffusion coefficient and molecular weight determinations. All samples used had molecular weights, translational diffusion coefficients, and particle-scattering factors in agreement with the data listed in Table II.

Gelation studies were conducted by raising the temperature of the cell holder using a circulating water bath. The sample temperature was determined using a thermocouple attached to the sample cell in the KMX-6 and using a thermometer placed in a fluid-filled well drilled into the cell holder of the Brice Phoenix. Studies indicated that the temperature within the scattering cell of the Brice Phoenix was within 0.2 °C of the cell holder temperature.

Samples were introduced at 10 °C into the scattering cell of the Brice Phoenix and Chromatix KMX-6 after filtration through 0.40- and 0.65-μ Millipore filters. No change in the Rayleigh factor was observed at 10 °C for periods of up to 8 h over the range of concentrations studied (0.05-0.50 mg/ml). Collagen concentrations were determined from measurement of the refractive index using a Chromatix KMX-16 laser differential refractometer and by amino acid analysis.

RESULTS

Previously (8) it was reported that acid-soluble rat tail tendon collagen in 0.01 M HCl after filtration through a 0.65- and 0.45-μ Millipore filters had a molecular weight of 805,000 and a diffusion coefficient of 0.450 × 10⁻⁷ cm²/s. After neutralization with phosphate buffer in the cold, this preparation aggregates as judged by the fact that it can no longer pass through a 0.45-μ filter; however, if the solution is first filtered through a 0.65-μ filter, it will then pass through a 0.45-μ filter. The filtered material now has a molecular weight of 476,000

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± 25,000 (see Fig. 1), a diffusion coefficient of $0.500 ± 0.05 \times 10^{-7}$ cm$^2$/s, and a particle-scattering factor at a wavelength of 436 nm and angle of 90° of 0.25 ± 0.04. Using theoretically generated particle-scattering factors shown in Table I and the calculated weight average molecular weight and Z-average diffusion coefficient for mixtures of monomers and dimers, it is possible to calculate the fraction of monomers ($M_r = 285,000$) and dimers ($M_r = 570,000$) in the cold neutralized collagen solution. Results of these calculations are shown in Table II and illustrate that all 3 physical measurements are consistent with the presence of about 35% by weight monomers and 65% by weight dimers. The concentrations used in subsequent experiments ranged from about 0.05-0.500 mg/ml.

Typical intensity measurements as a function of angle for a neutralized collagen solution at various heating times are shown in Fig. 2. Temperature measurements in the cuvette of the Brice Phoenix suggest that it takes about 10 min to heat the sample to 26 °C from 10 °C during which time little or no changes can be measured in the intensity of light scattered at angles between 30 and 120°. In the time interval between 10 and 20 min, the intensity of scattered light is seen to rise significantly at angles less than 60°, while it remains almost unchanged at an angle of 90° (see Fig. 2). During the time interval between 20 and 23 min, the intensity at low and high angles increases rapidly. As shown in Fig. 3, the intensity time curve at 90°, which is equivalent to a turbidity-time curve, is characterized by the classical lag and growth phases, whereas...
intensity-time measurements extrapolated to zero degrees exhibit only a thermal lag period. The ratio of the slope of the curve observed at 90° to the slope of the curve extrapolated to 0° is 0 during the period between 10 and 20 min and about 0.350 for times between 20 and 35 min.

Molecular weight measurements at 26°C and a scattering angle of 4° using a Chromatix KMX-6 light-scattering device indicate that the molecular weight increases from 476,000 to 930,000 during the first 20 min (see Fig. 4B) and 930,000 to 4,000,000 from 20 to 35 min as shown in Fig. 4A. After 35 min, the molecular weight increases rapidly, going from 4,000,000 to about 22,000,000 in the next 10 min (see Fig. 4A). Large increases in slope occur at molecular weights of about 930,000 ± 100,000 (3.26 ± 0.35 molecules) and 4 ± 1 × 10^6 (14 ± 3.5 molecules).

**DISCUSSION**

Classical light scattering as well as intensity fluctuation spectroscopy or laser light scattering (5, 12–15) has been used to study several biological assembly processes. Earlier laser light-scattering results on type I collagen (5) indicated that although the intensity of scattered light at 90° remained constant during the turbidity lag phase, the translational diffusion coefficient was observed to decrease from that of a single molecule (0.800 × 10⁻⁷ cm²/s) to about 0.36 × 10⁻⁷ cm²/s. It was suggested that the absence of intensity changes at an angle of 90° was only possible if aggregation occurred linearly. Theoretical diffusion coefficient calculations indicated that a 4D staggered trimer had a diffusion coefficient of 0.36 × 10⁻⁷ cm²/s consistent with the observed value of the diffusion coefficient measured at the end of the constant intensity period. In addition, it was observed that the intensity at 90° increased rapidly as the diffusion coefficient continued to decrease to a value of less than 0.20 × 10⁻⁷ cm²/s.

Although previous electron microscopic studies (4) suggested that discrete aggregates form during the turbidity lag phase, other solution studies (3) were unable to detect these changes. It was, therefore, important to reinforce earlier findings (5) by showing that another property besides the diffusion coefficient changes during the lag period.

The intensity of scattered light is perhaps the easiest physical parameter to follow during the lag period without disturbing the process of fibrillogenesis. At an angle of 0°, the intensity is proportional to the molecular weight and can be used as an assay of aggregation during the lag phase. These studies were conducted using neutralized acid-soluble rat tail tendon collagen which based on its molecular weight (476,000), translational diffusion coefficient (0.500 × 10⁻⁷ cm²/s), and particle-scattering factor (0.250) has a weight composition of 35% monomers and 65% dimers (see Table II). Measurement of the intensity of scattered light at different angles during heat gelation (see Figs. 2 and 3) demonstrated that although the intensity at 90° remains essentially constant after the sample reaches equilibrium (times between 10 and 20 min), the intensity at low angles increases by a factor of up to 2. It has been recently pointed out (10) that the turbidity is proportional to the intensity of scattered light at an angle of 90° and when plotted as a function of time during gelation (see Fig. 3) resembles classical turbidity-time curves. More important, however, is that extrapolation of a series of angular intensity measurements to zero degrees as a function of time leads to another sigmoidal shaped curve which is proportional to the molecular weight. It is quite obvious by comparing these 2 curves that after the thermal lag period is completed, which requires about 10 min, the intensity extrapolated to 0° increases even though that at 90° it is constant (see inset Fig.

3). From these results and previous arguments (5, 8), aggregation during the lag phase must be linear.

In order to measure the actual molecular weight during the lag phase, samples of type I collagen were heat gelled in the Chromatix KMX-6 laser light-scattering device. Results of these experiments indicate that the molecular weight at the end of lag phase (20 min at 26°C) is 930,000 or about 3 molecules which is consistent with the previous proposal of a 4D staggered trimer (5). It should be further emphasized based on Figs. 3 and 4B that the turbidity lag phase is terminated once the molecular weight exceeds 930,000. This is not consistent with the recent lag phase model of long thin filaments proposed by Williams et al. (6) and Gelman et al. (7) based on electron microscopy and laser light scattering (15). The interpretation of their data is to a large extent dependent on electron microscopy. It is very difficult to assure that no additional aggregation occurs during specimen preparation for electron microscopy unless several precautions are taken (16) or that the filaments one sees actually represent the bulk of the material and not a small fraction. It is likely that aggregates previously seen (4, 6, 7) represent products which are formed during the rapid growth phase. Brennan and Davison (17) have recently concluded that the lag phase involves reaction of aldehydes on a small fraction of molecules since they could not observe a viscosity change in solutions in which nucleation promoting structures were stabilized. Their findings suggest that linear growth does not require the formation of cross-links and that linear aggregates may dissociate under the shear stresses developed in a viscometer.

An important question that cannot be answered based on these data relates to the mechanism preventing lateral growth before an aggregate slightly less than 3 molecules long is formed. The simplest explanation is that lateral aggregation requires supramolecular coiling and that a linear aggregate of 846 nm in length (12.4D where 4.4D = 300 nm) is required to make a single turn or a stable number of turns of the helix. It is also possible that the overlap of NH₂- and COOH-terminal ends modifies the backbone rotational movement of the triple helix which facilitates lateral aggregation. These interpretations as well as what follows are consistent with the two-dimensional D-staggered model (18) and the Smith 5-stranded twisted microfibril (19) as well as the quasi-hexagonal model of fibril structure (20).

After the weight average molecular weight reaches 930,000 (t = 20 min), there is a second discontinuity in the rate of increase of molecular weight which occurs at an average molecular weight of about 4.0 ± 1.0 × 10⁶, 14 ± 3.5 molecules, which is equivalent to approximately 5 trimers. Previous studies suggest that the diffusion coefficient during the initial phase of lateral growth is about 0.20 × 10⁻⁷ cm²/s (5), which is close to the diffusion coefficient of a D-staggered lateral aggregate of 5 linear trimers. If the 5 linear trimers are D-staggered and twisted into a rope, then this aggregate is consistent with the microfibrillar subunit previously proposed. Due to the large experimental error in measuring the molecular weight as well as the weighting of the molecular weight toward the high molecular weight species, it is quite possible that this aggregate may contain some dimers or tetramers. However, regardless of the exact number of molecules in this unit, this is the first kinetic evidence in vitro favoring the existence of such a unit as a building block for the fibril. Further growth involves both concurrent linear as well as lateral growth since the ratio of the change in intensity at an angle of 90° to that at 0° is about 0.350 which is between that observed for linear (ratio = 0) and lateral growth (ratio = 1).

It is important to emphasize that the observed molecular weight is a weight average and is weighted to the higher
molecular weight species. For this reason, it is not possible to
rule out monomer addition to a growing aggregate during
lateral growth. The model of lateral growth that is presented
may apply only to the higher molecular fraction of aggregates.

Another observation made during the course of these ex-
periments related to the concentration and temperature de-
pendence of the lag phase. It is known that the rate of fibril
formation (21, 22) is very sensitive to the concentration of
phosphate ions with lag times as short as several minutes
having been previously measured. During the course of these
experiments, it was observed that in general for collagen
concentrations up to 0.405 mg/ml, the lag or initial linear
growth time was independent of concentration at an ionic
strength of 0.2. (See Table III). Further studies at temperatures
between 17 °C and 27 °C indicated that the time for trimer
formation was also independent of temperature as seen from
data in Table IV. Since the general temperature and concen-
tration dependence of collagen fibril formation in vitro have
been well established (23–25), there are at least 2 possible
explanations for the lack of concentration and temperature
dependence of the lag phase over the ranges of these variables
studied in this report. Since turbidity and intensity measure-
ments are weighted in proportion to the molecular weight (9, 10),
the presence of even a small fraction of high molecular
weight material will markedly influence the observed turbidity
and change in turbidity. Many earlier studies of heat gelation
were performed on starting solutions (1, 21–25) which were
not characterized by a molecular weight determination. Com-
per and Veis (3) have shown that pepsin-solubilized rat skin
collagen has aggregates with a molecular weight of 4.5 × 106.
In general, it is recognized that most preparations of collagen
contain high molecular weight aggregates which even at low
concentrations could significantly dominate the rate of change
of the turbidity. For this reason, it seems appropriate to
question whether the temperature and concentration effects
previously observed apply to single molecules or aggregates.

A more likely explanation of the lack of temperature and
concentration dependence of the lag time relates to the second
viral coefficient. Previous studies on collagen in acid solutions
(8) suggest that collagen single molecules and aggregates
exhibit intermolecular attractive forces (negative second viral
coefficient), whereas in the presence of phosphate, the second
viral coefficient (slope of Fig. 1) is close to zero. In the absence
of strong intermolecular interactions between collagen mol-
cules, the effects of increased concentration and temperature
on the collision frequency would be less apparent especially
since the observed lag times are short and the standard
deviations large.

In summary, collagen fibrillogenesis in vitro initiates by the
formation of linear dimers and trimers which occur during the
lag phase of turbidity-time curves. Once trimers are formed
terminating the lag phase, rapid lateral aggregation involving
about 5 linear trimers occurs to form an intermediate consistent
with the twisted ropes proposed based on x-diffraction
modeling (26, 27) and other considerations. This unit can grow
either by linear or lateral addition of other units although the
exact mechanism is beyond the scope of these studies.

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Supplement

To: Type I Collagen Fibrillogenesis In Vitro: Additional Evidence for the Assembly Mechanism

By: Frederick H. Silver

Experimental and Theoretical Methods

Intensity Measurements

Intensity measurements were made at a wavelength of 436 nm using a Perkin-Elmer model 402 spectrophotometer equipped with a 1-cm cell and a photomultiplier tube. The spectra were corrected for calibration to within 0.1% relative intensity (from a calibrated attenuator to 436 nm). Intensity measurements were conducted in a cell held at 20°C, and the intensity of scattered light was measured at angles between 30° and 120°. The intensity was subtracted from the scattering intensity of the solvent at an intensity of 100° and 120° was within limits set by day to day measurements.

During heating, the intensity was measured as a function of time and angle for a fixed value of the beam intensity. High values of intensity (the ratio of intensity of scattered light at a particular angle to that at the wavelength of 436 nm) were obtained using the methods of Doty and Steiner [10, 11]. Initial estimates reported elsewhere [10] have been extended and are shown in Table 1. At values of Q greater than 7.1, P(11) becomes dominant and with further increase in Q, the intensity is expected to increase with increasing values of Q. Values of P(11) measured using the methods of Doty and Steiner are probably correct for Q greater than 7.1. Using the Rayleigh-Debye approximation and the methods discussed in reference 11, P(11) has been calculated for Q up to 10.8 and is also shown in Table 1. Both methods give similar values of P(11) for Q less than 10.8, and the agreement is satisfactory using the Rayleigh-Debye relationship for P(11), as expected.

Theoretical Values of $R_0$, $D_0$, and $P(0)$

The weight average molecular weight, intensity and average diffusion coefficient and particle scattering factors were calculated assuming a molecular weight of 291,000 and 577,000, a translational diffusion coefficient of 0.500 and 0.500 $\times 10^{-7}$ cm²/sec and a refractive index of 1.356 and 1.356 for monomers and 2D staggered dimers, respectively, using equations 1 through 3.

$$ R_0 = \frac{L}{\alpha} $$

$$ D_0 = \frac{L}{\alpha^2} $$

$$ P(0) = \frac{L}{\alpha^3} $$

where $L$ is the weight fraction of the 1st species.

Results of these calculations are shown in Table 2 along with the experimental measurements.

Determination of the Concentration and Temperature Dependence of the Linear Growth (Lag Time)

The lag time was defined as the required time for the molecular weight to increase to 10,000 as determined by a KMX-6 laser light scattering device. All molecular weights were obtained from the inverse of $R_0$, where $R_0$ and $\alpha$ are as defined above. The weight concentration dependence for polyacrylamide was performed by varying the concentration dependence and the weight concentration dependence for polyacrylamide was performed by varying the concentration dependence and is shown in Tables 1 and 2 respectively.

Table 1

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Particle Scattering Factor P(0) for Rods</th>
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<td>$D_0$</td>
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Table 2

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Table 3

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<td>Concentration</td>
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Table 4

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