Collagen Fibril Formation

EVIDENCE FOR A MULTISTEP PROCESS*

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If fibril formation in a cold collagen solution (0.1 mg/ml) is initiated by warming to 26°C for 10 min, the temperature can be reduced to 4°C for up to 40 min during the turbidity lag period (and then returned to 26°C) without affecting the reaction half-time of 125 min. Any additional time at 4°C increases the half-time by the same amount. The product present after 50 min at 26°C, or 10 min at 26°C followed by 40 min at 4°C, is long thin filaments with minimal diameters of 2 to 4 nm. Therefore, fibril formation in vitro requires at least three steps. The first step, initiation, involves a temperature-dependent change which leads to an unidentified intermediate. The second step is linear growth of filaments by a process that is apparently temperature-independent. The third step, which is associated with the turbidity change, is lateral association of filaments by a temperature-dependent process. The reaction times of both the second and third steps are inversely proportional to collagen concentration suggesting that both linear and lateral growth occur by accretion. If there is a nucleation step, it is not rate-limiting. However, it is likely that there is a temperature-dependent critical filament length for lateral assembly.

Reduced collagen (unable to cross-link) behaves the same way except that the reaction half-time is 210 min and the turbidity increase can be reversed by cooling, yielding filaments indistinguishable from those formed in Step 2. The longer half-time and a very small apparent critical concentration suggest that Step 3 involves a series of stages leading to an increasingly stable fibril. Covalent cross-linking may increase the net rate of lateral assembly and decrease the critical filament length by decreasing the rate of disassembly in the early stages. The filaments obtained by cooling are stable at 4°C but at 26°C they reassemble and form native fibrils again. Their structure is not known, but they may be the 5-fold helical microfibrils proposed from other evidence.

We have previously investigated conditions for in vitro formation of collagen fibrils and have selected optimal conditions to obtain native banded fibrils from a solution of rat tail tendon collagen (1). The collagen was prepared so as to be largely free of aggregates and to have intact nonhelical ends (2). Kinetic studies with this system showed that the turbidity lag and growth periods characteristic of fibril formation cannot be equated to a simple nucleation-growth process (1). Rather, turbidity is a measure of a process with a rate, defined as the reciprocal of the half-time, that is directly proportional to concentration suggesting growth by accretion. Furthermore, the apparent critical concentration was found to be very small and possibly zero. Based on the microfibril model of the collagen fibril (3-6), we proposed that turbidity may be predominantly a measure of lateral association of microfibrils, which must form during the turbidity lag period and are too small (thin) to be seen by turbidity.

We have continued these studies using electron microscopy and turbidity to follow fibril formation. We have investigated two questions. First, what is the nature of the assembly during the turbidity lag period? We have been able to use temperature manipulation to separate turbidity lag and growth periods. Second, how does covalent cross-linking, which is known to make the overall process irreversible, participate in fibril formation? We have used reduced collagen, which cannot cross-link, for this purpose.

METHODS

Collagen—Soluble collagen was prepared from rat tail tendons by the method of Chandrakasan et al. (3) and characterized as previously described (1). Collagen was reduced with sodium borohydride as described by Robbins and Bailey (7) except that reduction was allowed to proceed for a longer time. Collagen at 3 mg/ml in 0.005 M acetic acid was diluted at 5°C with an equal volume of the Tes'/phosphate/NaCl buffer used for fibril formation (1). For each 10 ml of collagen solution, 1.6 mg of sodium borohydride was added with stirring. The same amount of sodium borohydride was added again at 30 and at 60 min. After an additional 30 min, the reaction mixture was dialysed exhaustively against first 0.1 M acetic acid and then 0.01 M acetic acid at 5°C and lyophilized. The criterion used for completeness of reduction of aldehydes was reversibility of the turbidity increase associated with fibril formation (see under “Results”). The reduced collagen was recharacterized as before (1) and shown to have the same tyrosine content (as a measure of intact nonhelical ends), melting profile, and chromatographic behavior as the nonreduced collagen.

Electron Microscopy—The same methods were used as before (1). When samples were taken at 4°C, they were negatively stained at room temperature. In some cases the negative staining procedure of Valentine et al. (8) was used.

Fibril Formation—The same method was used as before (1), adding double strength Tes/phosphate/NaCl buffer to an equal volume of an acidic solution of collagen to give the following final conditions: pH 7.3, 30 mM Tes, 30 mM phosphate, NaCl to 0.225 ionic strength. The solution was warmed to 26°C to initiate fibril formation, and turbidity was recorded as optical density at 313 nm. The collagen concentration was 0.1 mg/ml unless otherwise noted. In some experiments, the temperature was changed during the course of the experiment by switching between two circulating water baths, one set at 26°C and the other set at 4°C. Temperature equilibrium was reached in less than 1 min.

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1 The abbreviation used is: Tes, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.
RESULTS

Turbidity Assay—The total turbidity change, $\Delta h$, and the time to reach $\Delta h/2$, $t_{1/2}$, were measured as before (1). Turbidity is proportional to amount of product as previously discussed (1). We also calculated the time to complete the turbidity lag period, $t_{lag}$, and the half-time of the turbidity increase, $t_{1/2} = t_{lag}$. Since the slope at $t_{1/2}$ is independent of collagen concentration ($\Delta h$ normalized) and of temperature (1), it was convenient to calculate $t_{lag}$ as $log t_{lag}$. We defined $log t_{lag}$ as the point where a line representing the slope at $log t_{1/2}$ intersects the turbidity baseline (Fig. 1). If the slope is approximated from the values of $log t_{1/4}$ and $log t_{1/4}$, defined as log time at $\Delta h/4$ and $3\Delta h/4$, it is apparent from Fig. 1 that $log t_{lag} = log t_{1/2} - (log t_{1/4} - log t_{1/4})$, or $log t_{lag} = log (t_{1/2}/t_{1/4}/t_{1/4})$; therefore, $t_{lag} = t_{1/2}t_{1/4}/t_{1/4}$. Values of $t_{lag}$ were either determined graphically (Fig. 1) or calculated from this last equation.

Turbidity Reversibility—Typical turbidity curves for nonreduced and reduced collagen are shown in Fig. 2. Two differences were seen. First, the time to reach $t_{1/2}$ was longer for reduced collagen. Second, if after fibril formation was complete at $26^\circ C$, the temperature was reduced to $4^\circ C$ for 12 h or more, the turbidity of fibrils from nonreduced collagen decreased less than 5% while the turbidity of fibrils formed from reduced collagen decreased 85 to 95%. There was some variation from sample to sample probably reflecting differences other than 4°C and 26°C have not been tried nor have other conditions been varied, so a more complex dependence on temperature has not been ruled out. In any case, the results show a distinct difference between very early and later events occurring during the turbidity lag period.

Data from a number of similar experiments using nonreduced and reduced collagen are shown in Fig. 4. Values of $t_{1/2}$ were plotted as a function of time at $4^\circ C$ plus the initial 10 min at $26^\circ C$. The lines were approximated by assuming a slope of 1.0 for the longer times at $4^\circ C$ and a slope of zero for the shorter times at $4^\circ C$. The data fit these lines within experimental error.

Temperature Dependence at Early Times—If collagen solution and buffer were mixed at $4^\circ C$ and kept at $4^\circ C$ for several hours before warming to $26^\circ C$, there was no change in $t_{1/2}$ measured from the time the temperature was raised (not shown). Fibril formation does not occur in the cold. However, if fibril formation was initiated by a short time ($10$ min) at $26^\circ C$, the reaction mixture could be cooled to $4^\circ C$ for up to 40 min for nonreduced collagen and 55 min for reduced collagen without affecting the value of $t_{1/2}$ or the shape of the turbidity change. Longer periods of cooling increased $t_{1/2}$ about the same amount as the time beyond 40 (or 55) min, but the shape of the turbidity increase was unaffected. This is shown in Fig. 3 for nonreduced collagen. Whatever is happening during the turbidity lag period is apparently independent of temperature. However, temperatures other than $4^\circ C$ and $26^\circ C$ have not been tried nor have other conditions been varied, so a more complex dependence on temperature has not been ruled out. In any case, the results show a distinct difference between very early and later events occurring during the turbidity lag period.

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FIG. 4. Values of \( t_{1/2} \) obtained with reduced (●) and nonreduced (○) collagen cooled to 4°C after an initial 10 min at 26°C. The abscissa shows the time at 4°C plus the 10 min at 26°C. Conditions as in Fig. 2.

FIG. 5. Turbidity values at \( \Delta h/4 \), \( \Delta h/2 \), and \( 3\Delta h/4 \) for nonreduced (○, □) and reduced collagen (●, ▧) plotted as a function of log time. The temperature for fibril formation was 26°C throughout (○, ●) or was 26°C for 10 min, 4°C for 40 min, and 26°C thereafter (□, ▧). Conditions as in Fig. 2.

Collagen concentration is shown in Fig. 6. The data for nonreduced collagen are those previously obtained (1). Reduced collagen showed a very similar type of dependence but displaced toward larger values of \( t_{1/2} \). The power dependence was obtained from the slope of a line through the data expressed as \( \log (1/t_{1/2}) \) versus log concentration (Fig. 7). As for nonreduced collagen (1), a slope of 1.0 was obtained. Therefore, the rate expressed as the reciprocal of the half-time is directly proportional to collagen concentration whether reduced or not. This rate applies to the overall process. If we assume that Steps 2 and 3 are consecutive and since Step 1 is rapid (see “Discussion”), \( t_{1/2} \) can be divided into \( t_{lag} \) and \( t_{lag} - t_{lag} \) to give measures of individual rates for steps 2 and 3, respectively. When this was done and \( \log (1/(t_{lag} - t_{lag})) \) and \( \log (1/t_{lag}) \) were plotted as a function of log concentration, both gave slopes of 1.0. The data for \( \log (1/t_{lag}) \) are shown in Fig. 7.

When the total turbidity change, \( \Delta h \), was plotted against initial collagen concentration, points for nonreduced and reduced collagen fell on the same line (Fig. 8). As previously discussed (1), these data show that the apparent critical

nonreduced collagen and about 65 min for reduced collagen (Fig. 4). The difference was small but experimentally reproducible.

Values of \( t_{lag} \) were also calculated from the same data. For samples not cooled or cooled less than 40 (or 55) min, \( t_{lag} \) averaged 80 min for nonreduced collagen and 160 min for reduced collagen. The slopes of the turbidity change plotted as a function of log time were the same for reduced and nonreduced collagen (Fig. 5). This result could be fortuitous but suggests that although the rate of assembly is different, the mechanism, in particular the relationship between the turbidity lag and growth periods, is not (see under “Discussion”).

If the initial time at 26°C was increased, the length of time at 4°C that would not change \( t_{1/2} \) was decreased by the same amount (not shown) as expected from the temperature independence of Step 2. We did not investigate times less than 10 min for the initiation step.

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nonreduced collagen could not be distinguished. When fib\(\)\
from reduced and nonreduced collagen. For re-
duced collagen, samples were also taken after turbidity revers-
(overnight at 4°C after reaching the turbidity plateau) and
after warming to 26°C to form fibrils a second time. Examples
of micrographs of reduced collagen taken at the four time
points are shown in Fig. 9. In some cases an additional cycle
of cooling and warming was monitored.
At end of lag, the only product seen was thin filaments (Fig.
9a) except for occasional clumps of an unidentified contami-
nant which is usually present in our samples (1). The filaments
obtained from both reduced and nonreduced collagen were
indistinguishable, and it did not matter whether the tempera-
ture had been 26°C throughout or 26°C for 10 min followed
by 40 or more min at 4°C. The filaments were very long and
had varying diameters from the limit of resolution, about 2
nm, to 10 nm or more. However, close examination (Fig. 10)
of the wider filaments showed them often to be composed of
thinner filaments which course among bundles in an irregular
manner resulting in a network appearance. Measurements of
filaments that appeared to be single gave apparent diameters
of 2 to 4 nm. Some were narrow enough to be single molecules
(1.5 nm), but they were generally wider. No periodic structure
was visible. Ends of filaments could be found but were difficult
to identify with certainty.
At the turbidity plateau, native banded fibrils were obtained
as previously described (1). Fibrils from reduced (Fig. 9b) and
nonreduced collagen could not be distinguished. When fibrils
made from reduced collagen were kept at 4°C overnight or
longer, filaments were again obtained (Fig. 9c). They appeared
to be identical with the filaments present at the end of lag
except for the occasional presence of thin, banded fibrils
accounting for the incomplete (85 to 95\%) reversal of turbidity.
Warming again produced native banded fibrils (Fig. 9d). Dis-
persal to filaments and reassembly to native fibrils could be
repeated at least an additional time without any change in
morphology.
To rule out the possibility that the filaments were artifacts
of drying, the Valentine (8) staining procedure was used for
some specimens. In this procedure, stain mixes with the
protein while still in suspension which fixes it and would
prevent, or at least alter the nature of, any aggregation during
subsequent drying. Identical micrographs were obtained using
the standard and the Valentine methods.

**DISCUSSION**

As reviewed in the introduction, we have previously sug-
gested that fibril formation in vitro is dominated by linear
growth in the turbidity lag period and lateral assembly during
the turbidity increase (1). Our present results support and
extend this model. We can now identify three steps which
have the following characteristics.

*Step 1, Initiation*—Soluble collagen in neutral solution and
moderate ionic strength is stable at 4°C for at least several
hours but at 0.1 mg/ml and 26°C it is rapidly (10 min or less)
converted to an intermediate form which can assemble. We
have no information about the nature of this form except that
its formation is temperature-dependent. The electron optical
results of Trelstad et al. (11) suggest that in their experiments
an intermediate aggregate is formed which may be a short
microfibril or small bundle of short microfibrils. If an inter-
mediate aggregate is involved, the temperature dependence
requires that it be assembled by a different mechanism than
its growth (Step 2). A possible explanation is that its assembly
involves nucleation. However, there is no evidence for nuclea-
tion and some evidence against it (see below).

*Step 2, Linear Growth*—Once the initial intermediate is
obtained it spontaneously grows by a process that is appar-
tently temperature-independent. The temperature independ-
ence may not reflect the real nature of the process since it
could result from an approximate balance of two processes
with opposite temperature dependencies. Growth must be
entirely or predominantly linear since there is no change in
turidity, and electron microscopy of the completed product
of Step 2 shows only long thin filaments. The thinnest ones
we see have diameters in the range of 2 to 4 nm. The simplest
explanation of the micrographs is that the 2 to 4 nm filament
is the limiting width and that these filaments are often loosely
associated. This diameter is consistent with the filaments
being 5-fold helical microfibrils, but additional studies are
needed to further characterize them.

The mechanism of growth during Step 2 is not known. If
the intermediate formed during Step 1 is a short microfibril
or small bundle of short microfibrils (11) and the microfibril
is a 5-fold helix of D-staggered collagen molecules in which all
molecules are equivalent (3–6), NH\(_2\) termini of one interme-
diate could add to COOH termini of another to form long
continuous filaments as suggested by Trelstad et al. (11).
Other possible mechanisms are continuing addition of mono-
er to a small number of initial intermediates or linear growth
of some of the intermediates at the expense of others by
recrystallization at filament ends. Whatever the mechanism,
it must fit our observation that the rate of Step 2, measured
as the reciprocal of \(t_{lag}\) is proportional to the initial collagen
concentration. This would seem to rule out addition of mono-
er following nucleation and probably most mechanisms
where the initial intermediate is formed by a nucleation step
since the rate would be expected to be proportional to some
higher power of concentration than one (12). If nucleation is
occurring, for some reason it is not rate-limiting. Without
some probe of early events, we can only speculate on this
manner.

Contrary to our interpretation, Comper and Veis (13) sug-
gest that the turbidity lag period is a nucleation phase since
they find no change in viscosity or molecular weight monitored
by light scattering and sedimentation equilibrium during the
Fig. 9. Samples of reduced collagen taken at various times during fibril formation and reversal (see Fig. 1). Samples were negatively stained with 1% phosphotungstic acid, pH 7.4. Magnification × 25,000. a, filaments observed at end of turbidity lag (60 min). An area from the top center is shown enlarged in Fig. 10. b, fibrils observed at turbidity plateau (400 min). The periodicity and the fine structure is similar to that of fibrils formed in vivo. c, filaments obtained by cooling fibrils (1100 min). A thin banded fibril can be seen near the bottom showing incomplete dispersal to filaments. d, fibrils obtained by rewarming filaments (1400 min). They are at least as well ordered as in b.

The very small critical concentration and the temperature independence are consistent with Step 2 being essentially irreversible. However, a dynamic process with a measurable light scattering would not be expected to be sensitive to the length of long, thin rods (14).
affinity constant is not ruled out. It should be noted that the critical concentration we measure, and its inverse, the affinity constant, applies to fibril formation but not necessarily to filament formation since Step 3 may drive Step 2 to completion.

The apparent temperature independence suggests that the stabilizing forces in filament formation are in large part electrostatic, which was also proposed from an analysis of the surface features of the collagen molecule in a helical microfibril model (15). Step 2 proceeds in the cold, but it and Step 1 presumably are reversed by dilute acid or at high ionic strength and neutral pH since solvents of these types are used to prepare soluble collagen from fibrillar collagen.

Our results explain the observation of Wood and Keech (16) and Comper and Veis (17) that a collagen solution carried through the turbidity lag period and then cooled has "thermal memory" or contains "low temperature-stable nuclei" and forms fibrils more rapidly when warmed a second time. That is, in their experiments Step 2 was probably complete at the time of cooling, and cooling simply delayed the onset of Step 3.

**Step 3, Lateral Growth**—Warming of filaments formed in the cold or at 26°C produces native banded fibrils with an increase in turbidity, and, if cross-linking is inhibited, cooling again gives filaments with a decrease in turbidity. The process can be repeated and the fibrils formed the second time are as well ordered as those formed the first time. Electron microscopy shows that dispersal of filaments on cooling is not an active process (there is no swelling of fibrils) but rather is a passive separation presumably driven by thermal diffusion. The temperature dependence suggests that the forces involved are largely hydrophobic, which was also proposed from an analysis of the surface features of the collagen molecule in a helical microfibril model (15).

A high degree of cooperativity in lateral assembly is suggested by the observation that the product present at the end of the lag period is the same, and Step 3 begins at the same time, whether the sample has been cooled during the lag period or not. Furthermore, the sigmoid shape of the turbidity increase is independent of the time at 4°C. The 50 min for nonreduced collagen and 65 min for reduced collagen are quite sharply demarcated as the minimal times required to make filaments long enough to assemble laterally under the given experimental conditions.

Even stronger evidence for cooperativity is obtained from the following considerations. We showed previously that $t_{1/2}$ is temperature-dependent and gives an apparent heat of activation of 58 kcal/mol for fibril formation (1). We also showed that the slope at log $t_{1/2}$ is independent of temperature (1). It follows then that $t_{lag}$ is a constant proportion of $t_{1/2}$ and has the same dependence on temperature as $t_{1/2}$. However, we show here that the process occurring during the turbidity lag period is temperature-independent. This can only mean that $t_{lag}$ is not just a measure of the rate of Step 2, but must contain a temperature-dependent factor. This factor is most likely filament length. That is, there seems to be a critical length for lateral assembly which is strongly temperature-dependent.

Cross-linking—Covalent cross-linking occurs during Step 3, if preformed aldehydes are present, and presumably accounts for the faster net rate of lateral assembly by decreasing the rate of disassembly. However, since the critical concentration is very small for both reduced and nonreduced collagen, these results imply that Step 3 consists of a series of stages leading to an increasingly stable fibril, and covalent cross-linking affects primarily the early stages where disassembly rates would be significant. The longer $t_{lag}$ for reduced collagen by the same reasoning may reflect an increase in critical length. That is, if covalent cross-linking stabilizes the early stages of lateral assembly, the filaments need not be as long to associate. Alternatively, cross-linking may also occur during Step 2 and increase the net rate by decreasing the rate of disassembly as in Step 3. However, this explanation seems
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less likely than an effect on critical filament length since independent effects on Steps 2 and 3 would require that the observation of the same slope at log $t/t_0$ for reduced and nonreduced collagen is fortuitous. Furthermore, covalent cross-linking would probably be temperature-dependent, and we show the rate of Step 2 to be temperature-independent. Of course, cross-linking may occur during Step 2 and not affect the kinetics.

Tanzer et al. (10) and Comper and Veis (17) have looked at the kinetics of assembly of collagen treated with thiosemicarbazide to block cross-linking. They have reported that although the turbidity increase from fibrils made from thiosemicarbazide-treated collagen is reversed by cooling, the rate of the initial turbidity increase is not affected, unlike our results with reduced collagen. We attempted to resolve this difference by treating our collagen with thiosemicarbazide under the same conditions as used by Tanzer et al. (10) and studying its kinetics of assembly. However, turbidity reversal on cooling for 24 h after fibrils were formed was only 45 to 50% indicating incomplete blockage of cross-linking. This may be because thiosemicarbazide makes a more stable adduct with the αβ-unsaturated aldehyde, allysine aldol, present in β components, than with the simple aldehyde, allysine, present in α chains (10); our samples contain very little β component but probably are rich in allysine since the nonhelical ends where the aldehydes are located are intact. Therefore, the different results obtained by us and Tanzer et al. (10) and Comper and Veis (17) may be ascribed to a higher potential for cross-linking of our untreated collagen and, therefore, a larger difference between treated and untreated samples. Furthermore, Tanzer et al. (10) and Comper and Veis (17) used higher collagen concentrations and temperatures than we did, which, because of the faster rates, could make differences more difficult to detect.

Filaments—The filaments seen at the end of Step 2 and those produced by cooling native fibrils made from reduced collagen seem to be the same. No periodic structure can be seen in them. However, since they reversibly form n-periodic native fibrils by lateral association, it is reasonable to propose that the filaments themselves have a d-period (or an nd period, where n is a small integer). The limiting diameter of about 2 to 4 nm allows only a few structures of this type, one being the 5-fold helical microfibril in which all molecules are equivalent (3–6).

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