Collagen Fibril Formation

OPTIMAL IN VITRO CONDITIONS AND PRELIMINARY KINETIC RESULTS*

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(Received for publication, January 18, 1978)

Soluble rat tail tendon collagen with intact nonhelical ends and largely free of aggregates was used to study fibril formation in vitro. The process was initiated by raising the pH and warming a cold solution of collagen. Fibril formation was monitored by turbidity and the product was examined by electron microscopy. Optimal conditions that will form fibrils similar to those observed in vivo are: a solvent containing 30 mM phosphate, 30 mM NaCl, and Na2CO3 to give an ionic strength of 0.225 at pH 7.3; temperatures between 20 and 30°C; and collagen concentrations between 0.02 and 0.5 mg/ml. Phosphate is required to obtain well ordered fibrils. Under other conditions polymorphic aggregates may form, including nonbanded filamentous forms and two types of D-periodic symmetric-banded fibrils, one of which has not been previously described.

Turbidity shows a lag period followed by a growth phase. The overall process is strongly temperature-dependent with an activation energy of 58 kcal/mol. The rate of fibril formation as measured by turbidity halftimes is directly proportional to collagen concentration. The apparent critical concentration is <7 mg/ml and could be zero. We conclude that turbidity is a measure of growth by accretion rather than nucleation-polymerization. Based on the microfibril model of the collagen fibril, it is possible that this growth process is dominated by lateral association of microfibrils. Assembly of monomer to microfibril would then occur during the lag period and involve intermediates too small to be seen by turbidity.

Type I collagen, the major structural component of skin, tendon, bone, and other connective tissues, is found in the form of fibrils with diameters of about 30 to 300 nm. The basic subunit of the fibril, the collagen molecule, consists of three polypeptide chains in triple-helical array forming a rod (300 subunit of the fibril, the collagen molecule, consists of three form of fibrils with diameters of about 30 to 300 nm. The basic tendon, bone, and other connective tissues, is found in the integral value, the density varies along the fibril accounting for molecules gives rise to a characteristic band pattern of light or 234 amino acid residues by sequence analysis. The staggered molecules, the microfibril, and an ordered packing of aligned and parallel microfibris (4, 5). The microfibril would have a diameter of about 4 nm. The diameter of a fibril then depends on the number of microfibrils in it, according to this model, and two sets of interactions (intra- and intermicrofibris) are required for its formation (6, 7).

The mechanism of fibril assembly in vivo is not understood but assembly can be induced in vitro starting with a defined solvent and a purified soluble collagen. Under some conditions fibrils are formed which are very similar or identical to native collagen fibrils produced in vivo. Early studies on systems of this kind have been reviewed by Wood (8). Although fibril formation is generally treated as a nucleation-polymerization process (8) driven by a positive entropy change in the associated solvated (9, 10), evidence for this model is not definitive. Difficulties arise because the process is highly sensitive to the source and method of purification of the collagen, to the solvent system employed, and to the conditions under which fibril formation is initiated. Furthermore, a variety of polymorphic aggregates in addition to the native fibril may form (4, 11, 12) for poorly understood reasons.

In the studies reported here, we attempt to surmount these problems by using highly purified rat tail tendon collagen prepared in such a manner that it consists largely of native monomer with intact nonhelical ends (13). These characteristics typically vary widely in collagen preparations and may be responsible for variability in results. In particular, it is likely that the collagen used in all early studies, and perhaps more recent ones, lacked at least the COOH-terminal nonhelical ends and may have been modified at the NH2-terminal ends because of the sensitivity of these regions to proteolysis, as discussed by Chandrakasan et al. (13). A variety of studies have shown that deliberate removal of these ends by proteolytic treatment alters the kinetics (8, 14, 15) and may change the structure of the product (16).

Our present purpose is to select experimental conditions under which native fibrils can be reproducibly formed from intact soluble collagen using stringent morphological criteria. We also present some preliminary kinetic results which show that assembly may be quite different from that observed for other proteins.

MATERIALS AND METHODS

Collagen—Soluble collagen was prepared from rat tail tendon by the method of Chandrakasan et al. (13) with the following modifications: (a) young rats (40 to 50 g) were used to further decrease the amount of aggregate present, and (b) salt precipitations of collagen from 0.5 M acetic acid were performed by adding the appropriate volume of 25% NaCl in 0.5 M acetic acid (w/v). The suspension was stirred 3 h and allowed to settle 3 h prior to centrifugation at 8000 x g for 30 min. The 3 to 4% salt cut was used for all experiments. Samples were stored as the lyophilized product in a sealed container.

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Collagen preparations were characterized by molecular sieve chromatography, CM-cellulose chromatography, proton NMR, and amino acid analysis as described by Chandrakasan et al. (19). Denatured samples contained 70 to 90% α chains, 10 to 30% β component, <1% γ component, and, at most, traces of higher molecular weight material. The tyrosine contents were in the range 3.5 to 4.5 residues/1000. Samples were also characterized by the melting profile determined by following optical rotation at 313 nm in a Cary 60 spectropolarimeter using a value for the specific rotation of -2300 at 4°C (native) or -825 at 43°C (denatured) (17). The stock was diluted to 0.20 to 0.21 mg/ml (unless otherwise indicated) with 0.005 M acetic acid. Dilutions stored at 5°C could be used for up to 2 weeks.

Solvents were prepared double strength from NaCl and a buffer. Concentrations of mono and dibasic phosphate at pH 7.3 were calculated from the acid dissociation constants given by Bates and Acree (18) for 25°C. The pH was adjusted with NaOH or HCl. Ionic strengths were calculated from the ion activities as described by Davies (19). Concentrations, ionic strengths, and pH values given under “Results” are the values after mixing with an equal volume of collagen solution. The solvent (double strength) selected for kinetic studies has the following composition in grams per liter: NaCl, 15.75; TeS, 13.76; Na₂HPO₄, 16.08; plus 1.4 ml of 10 N NaOH to pH 7.45 to 7.50. The buffer was filtered and stored at 5°C under sterile conditions.

Fibril formation was initiated by mixing equal volumes of collagen solution and buffer in an ice bath, checking that the pH was 7.3 to 7.4 (unless otherwise indicated), and introducing a portion into a water-jacketed cell (6-mm light path) at the desired temperature in a Cary 14 spectrophotometer. Turbidity was recorded continuously as the optical density at 313 nm. In some cases an additional sample was incubated at the same temperature in a water bath. Temperature was controlled to ±0.1°C. The sample reached equilibrium in less than 1 min.

Electron Microscopy—Shortly after the turbidity maximum was reached, a portion of the incubated sample was retrieved with a plastic pipette. A drop was placed on a carbon film supported on a 400-mesh copper grid which, after 30 s, was drained slowly with filter paper and then floated inverted on a large volume of freshly prepared 1% sodium phosphotungstate, pH 7.4, for 10 min. For those experiments done at different pH values, the pH of the stain was adjusted to the pH of the experiment. The grid was removed, drained slowly, and air-dried. The preparation was examined in a JEM 100B electron microscope operated at 100 kV and fitted with a liquid nitrogen-cooled decontamination device. Usually two identically prepared grids were examined and micrographs were taken at nominal magnifications of 30,000 and 60,000. Magnifications on prints were determined by assuming that the collagen periodicity is 67 nm (5).

**RESULTS**

**Turbidimetric Assay**

We define turbidity here as optical density under the conditions used. It is not simply light scattering from long thin rods, as is true for microtubules (20) and keratin filaments (21) since we find a wavelength power dependency of -1.3 (not shown) for completed collagen fibrils rather than the predicted value of -3.0 (22). Light scattering from collagen fibrils is apparently complex. In addition there may be a contribution from light absorbance resulting from a long light path created by multiple internal scattering centers within a fibril. However, Wood and Keech (23) and Gross and Kirk (24) have shown that optical density is proportional to the amount of sedimentable product and, as demonstrated below, optical density of fibrils formed is proportional to the initial collagen concentration. The proportionality constant may vary with the conditions, but we will assume it does not for present purposes, except in one case noted later.

The type of turbidity curve obtained is shown in Fig. 1. It

![Fig. 1](http://www.jbc.org/content/254/17/6579/F1.large.jpg)
is characterized by a lag phase where there is no detectable change in turbidity, a growth phase during which turbidity changes rapidly, and a plateau region where turbidity again remains constant. These curves can be characterized by the total turbidity change, $\Delta h$, and the time to reach $\Delta h/2$, $t_{1/2}$. In principle, the rate of change of turbidity in the growth phase could vary independently of $t_{1/2}$, in which case an additional parameter, such as the slope at $t_{1/2}$, would be needed. The slope can be approximated from the values of $t_{1/2}$ and $t_{1/4}$ (Fig. 1). Cassel et al. (25) have shown for collagen that the slope is independent of temperature when turbidity is plotted as a function of log time. We find that this is also true for different collagen concentrations if $\Delta h$ is normalized (see below). A constant slope is evidence that the mechanism of growth is the same.

If the temperature is returned to 4°C after the plateau region is reached, turbidity decreases no more than 5% overnight (Fig. 1). This irreversibility has been previously observed (26) and is known to be the result of spontaneous cross-linking involving lysine-derived aldehydes (27).

**Morphology**

Various polymorphic aggregates of collagen were seen. These have been described (4, 11, 12) except for one of the DPS forms (see below), but are briefly summarized here as seen in our experiments to facilitate description of our results. In all cases the samples were obtained shortly after the turbidity plateau was reached. We are able to interpret the morphology in terms of the proposed microfibril model of the collagen fibril as discussed in the introduction. However, other explanations are not ruled out.

Native-banded Fibrils—Reconstituted native-banded fibrils (Figs. 2B and 3A) are similar or identical to fibrils formed in vivo (Fig. 2A). When negatively stained, they show the D period consisting of a fine polarized band pattern superimposed on alternate light and dark regions. The fibrils are very long with ends only occasionally seen and have diameters of about 30 to 300 nm which is about the same range as rat tail tendon fibrils formed in vivo (28). When ends are seen they have a characteristic taper that is sometimes referred to as tactoidal. The filamentous substructure is ideally quite regular but often appears to be looser than in fibrils formed in vivo. A reconstituted fibril usually has a constant diameter throughout its length but tends to bend more readily than a fibril formed in vivo and sometimes bifurcates (or fibrils fuse) which is not normally seen in vivo. Under some conditions native-banding is evident but alignment is poor (Figs. 3B and 4C).

DPS Fibrils (Figs. 2, C and D, and 4A)—These have the same periodicity and general appearance as native-banded fibrils except that the band pattern is centrosymmetric. They may be difficult to distinguish from native-banded fibrils at low resolution. They are believed to contain normal microfibrils which, however, are packed antiparallel with a stagger (4, 29). We observe two different forms, which presumably arise from different stagger. We have not analyzed their patterns in detail but one (Figs. 2C and 4A, black arrow) is similar to that commonly observed with type I collagen (11, 16); it has been designated DPS III by Bruns (12). The other (Figs. 2D and 4A, white arrow) exhibits a narrower dark-staining region and has apparently not been previously described; we refer to it as DPS IV. Transitions between DPS III and native banding and from DPS III to DPS IV are sometimes seen across (but never along) a single fibril. The latter transition can be seen in Fig. 2D.

Filamentous Nonbanded Fibrils (Fig. 4D)—Like native-banded fibrils, these fibrils have diameters of 30 to 300 nm and are very long. The filaments in the substructure are

\[ 1 \] DPS I and II designate type II collagen DPS fibrils (12).
FIG. 4. Electron micrographs of negatively stained collagen fibrils obtained under nonoptimal conditions. Magnification, × 70,000. A, pH 8.5; native-banded fibrils are accompanied by both DPS III and IV (small arrows). An impurity can be seen in one place (large arrowhead). B, high ionic strength (0.3); fibrils have native banding but a different appearance (see text). C, high collagen concentration (0.5 mg/ml); native-banded fibrils are not tightly packed and are accompanied by other filamentous forms. The impurity can also be seen in several places in this micrograph (lower left center). D, high temperature (37°C); the fibrils are nonbanded or poorly banded and the filamentous substructure is very fine.
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similar to those in native-banded fibrils and may be normal microfibrils. Diameters obtained by measuring the thinnest filaments are in range 2 to 4 nm. The filaments are loosely packed and banding is absent or very weak. Native-banded and nonbanded fibrils may appear in the same field.

Other Filamentous Aggregates—Aggregates are seen that have a filamentous substructure similar to that in nonbanded fibrils but are characteristically narrower and are either shorter or entwined. There are several forms of these aggregates. One, seen in Fig. 4C with native-banded and poorly banded fibrils, has the appearance of a network of filaments and small bundles of filaments of varying diameters. In other cases the bundles have more uniform diameters in the range 20 to 40 nm but they are more entwined than filamentous nonbanded fibrils.

All collagen preparations contained an unidentified impurity which was seen as clumps of material associated with collagen fibrils when the sample was examined by electron microscopy after forming fibrils under optimal conditions. Examples can be seen in Fig. 4, A (large arrowhead) and C. It could not be detected by the other methods used to characterize the collagen. The contaminant did not affect the general morphology of the fibrils other than some localized distortion, and samples with very small to relatively large amounts of the impurity behaved the same with regard to kinetics. Samples were not used if the contaminant was prominent.

Buffers

Preliminary experiments were conducted to choose a buffer/salt mixture. The following buffers were tried (30 mM buffer, 150 mM NaCl, 0.1 mg/ml of collagen, 26°C, pH 7.3): Tes, 4-(2-hydroxethyl)-1-piperazineethanesulfonic acid (Hepes), 4-morpholinepropanesulfonic acid (Mops), cacodylate, veronal acetate, and phosphate. Phosphate produced the best native-banded fibrils, but pH control was inadequate. Higher concentrations of phosphate inhibited the rate of fibril formation (see below), so buffer mixtures were tried. Tes gave good pH control and, when included at different concentrations up to 30 mM, did not have any obvious effect on either the rate of formation or the structure of the product. The other buffers gave less satisfactory results, but were not studied in detail. Tes at 30 mM was therefore included in the solvent for all further experiments.

The effect of phosphate concentration at constant pH and ionic strength was examined in detail. The rate of fibril formation (Fig. 5) did not change significantly up to about 30 mM, above which increased rapidly. At phosphate concentrations near 30 mM the native banding appeared to be best and unincorporated filamentous aggregates were rare (Figs. 2B and 3A). DPS fibrils were extremely rare: only one was seen in several hundred fields. At low phosphate, banding was still evident but alignment and lateral packing were poorer (Fig. 3B). In the absence of phosphate, filamentous nonbanded fibrils were common. At high phosphate DPS III and IV fibrils (Fig. 2D) mixed with native-banded fibrils were seen as well as other filamentous aggregates. Phosphate at 30 mM was included in all further experiments.

Ionic Strength

The ionic strength was varied by changing the NaCl concentration. Over an ionic strength range of 0.15 to 0.30 at 26°C and 0.1 mg/ml of collagen there was an approximately 2-fold linear increase in (Fig. 6). At the lower end of the range the native-banded fibrils were loosely packed and accompanied by filamentous nonbanded forms. Banding was better in the middle of this range and fibril width was similar to that commonly seen in vivo (Fig. 3A). At higher ionic strengths very well packed native-banded fibrils were obtained but they had a finer substructure and a sheet-like appearance suggesting flattening (Fig. 4B). Negative staining was less effective as ionic strength increased and may have contributed to the different appearance. An ionic strength of 0.225 was selected for further experiments. A check of phosphate concentration at this ionic strength indicated that 30 mM was still optimal.

pH

The sensitivity of fibril formation to pH changes near neutrality was investigated. In the pH range 6.5 to 8.0 there was a small decrease in with increasing pH (Fig. 7). No
difference in morphology could be seen in the range 7.0 to 7.5. At pH 6.5 DPS III fibrils (Fig. 2C) were present in most fields. At pH 8.0, native-banded fibrils were common but showed surface distortion and loose lateral packing; numerous DPS III and IV fibrils were also present (Fig. 4A).

**Temperature**

The temperature at which fibril formation proceeded was varied from 12-37°C (Fig. 8). The sharpest absolute change in $t_{1/2}$ was observed in the range 20-30°C. There was no discernible change in fibril structure in this range and $\Delta H$ was constant. There is an apparent discontinuity in the $t_{1/2}$ data above about 35°C (Fig. 8) which may result from partial denaturation. When turbidity was plotted as a function of log time, the slope at $t_{1/2}$ was constant (Fig. 9), showing that only the rate is affected by changes in temperature (25). At lower temperatures most of the protein appeared as a mixture of filamentous aggregates with occasional thin native-banded fibrils. At higher temperatures nonbanded and poorly banded fibrils predominated (Fig. 4D). An Arrhenius plot (not shown) of the data in Fig. 8 showed anomalous behavior at high and low temperatures. The middle portion, where native-banded fibrils were produced, was linear and gave an activation energy of 58 kcal/mol of collagen.

**Collagen Concentration**

The dependence of $t_{1/2}$ on concentration was determined in the range 0.023 to 0.94 mg/ml. A plot of log (1/$t_{1/2}$) versus log concentration (Fig. 10) fitted with a least squares straight line gave a slope of 1.0. The rate of fibril formation as measured by turbidity is therefore proportional to collagen concentration raised to the first power.

In nucleation-polymerization reactions the amount of polymer formed, $c_{\infty}$, is related to the initial collagen concentration, $c_0$, by the expression $c_{\infty} = c_p + c_c$ where $c_p$ is the concentration of monomer in equilibrium with polymer or the critical concentration (30). If $\Delta H$ is proportional to $c_p$ (as discussed above), $c_c = m \Delta H + c_c$, where $m$ is a constant. When our data were plotted to obtain a value for $c_c$ (Fig. 11) a least squares line through all the points gave a value of $c_c$ not significantly different from zero. However, the relationship was not linear.
at lower concentrations. Extrapolation of the data at concentrations below 0.1 mg/ml using a least squares fit gave an apparent $c_c$ of $<7 \mu g/ml$.

An explanation for the nonlinearity may be found in the morphology of the product. Although there was no obvious difference in appearance of the fibrils obtained at low concentrations, the average diameter could have been smaller. Curvature would then result since turbidity will be a function of both fibril diameter and concentration, and extrapolation to zero concentration would give $c_c$ for the limiting fibril width. However, the value obtained for $c_c$ is maximal since the true value of $m$ at any point decreases with fibril width and is less than the apparent value of $m$. Other explanations for the nonlinearity are possible. The important point is that $c_c$ is very small and could be zero.

At high concentrations, above about 0.5 mg/ml, native-banded fibrils were accompanied by increasing amounts of mixed filamentous aggregates (Fig. 4C). The possibility that the polymerization mechanism might be different at different concentrations was examined by plotting turbidity (normalized to a value of 1 in the plateau region) as a function of log time. As with temperature, the slope at $t_{1/2}$ was constant in the range 0.947 to 0.94 mg/ml (Fig. 12), indicating that only the rate of assembly was affected. At lower concentrations the experimental error was too large to give useful results.

**DISCUSSION**

Our experiments show that reconstituted collagen fibrils which are very similar to fibrils formed in vivo can be obtained reproducibly from purified rat tail tendon collagen in a solvent consisting of 30 mM Tes, 30 mM phosphate, and NaCl to give a total ionic strength of 0.225 and pH 7.3 in the temperature range 20-30°C and at collagen concentrations up to about 0.5 mg/ml. Small changes in ionic strength and pH are not critical, but large changes in these variables, a temperature or concentration outside the indicated range, or a higher or lower phosphate concentration can markedly affect the morphology of the product. The conditions selected by us as optimal are presumably not the only set of conditions which will produce native fibrils in vivo, but they are experimentally convenient and not markedly dissimilar from in vivo conditions.

The simplest explanation of the polymorphic aggregates seen, based on the microfibril model of collagen (4, 3), is that two types of packing errors can occur. One is that microfibrils sometimes pack in an antiparallel manner. Two relationships between antiparallel microfibrils are possible, forming DPS III and IV. This error is associated in our experiments with high and low pH or high phosphate concentration. Even under these conditions most fibrils are native-banded. When DPS III banding is seen there is never a transition to native-banding along the fibril but there sometimes is across a fibril. This observation suggests that microfibrils in a completed fibril are very long, perhaps as long as the fibril. This possibility is also consistent with the observation that a given fibril formed under optimal conditions has a constant diameter for its full length unless it bifurcates. The second packing error is that microfibrils sometimes do not associate and align properly, forming nonbanded fibrils and filaments. This error is associated primarily with low or absent phosphate, high temperature, high collagen concentration, or low ionic strength. Even under optimal conditions in vitro reconstituted fibrils are not quite as well ordered as fibrils formed in vivo.

Phosphate appears to play a critical role in fibril formation beyond its capacity as a buffer. Its inhibition of the rate of fibril formation is well known (31). However, its role cannot be related to a rate effect since 30 mM is sufficient to produce well ordered native-banded fibrils without affecting the rate. The nature of its interaction with collagen is not known.

In a nucleation-polymerization process, $c_c$ is the concentration below which nuclei are unstable and polymerization cannot occur. The apparent $c_c$ value of $<7 \mu g/ml$ that we observe for collagen assembly is considerably smaller than that found for other protein assembly systems. For example, actin (21) and tubulin (22, 32) have $c_c$ values of 50 and 200 to 2000 $\mu g/ml$. The inverse of $c_c$ is related to a rate effect since $30x10^{-6} M^0$. This large value suggests that collagen assembly may not be adequately described by a nucleation-polymerization mechanism. An alternative is that assembly involves an irreversible step, such as a conformational change or a chemical reaction. Covalent cross-linking is a possibility since it is responsible for the irreversibility of the final stage. However, Comper and Veis (15) have presented evidence that cross-linking is a late event which does not alter early steps. Additional studies are required to clarify these points for our system.

A power dependence of $t_{1/2}$ on collagen concentration of one is also not typical of a nucleation-polymerization process. In the Oosawa model (30) this value is half the number of monomer units in a nucleus. For example, in keratin assembly a value of approximately 3 is observed (21) and in hemoglobin S assembly 30 to 40 is obtained (33). Reflecting the multicentric nature of the nuclei in these processes. A power dependence of one suggests simple growth by accretion.

We interpret these data as showing that the turbidity lag and growth periods in collagen assembly cannot be equated to nucleation and polymerization as defined by Oosawa (30). A possible explanation of what is occurring is suggested by the microfibril model of the collagen fibril. The lag period may be dominated by linear growth of microfibrils, which are too narrow to be seen by turbidity, while the turbidity increase measures primarily lateral assembly of microfibrils to fibrils. The lag stage could be a simple growth process as indicated by turbidity measurements. The large affinity constant (or irreversible step) would apply to the earlier stage. These two stages are consistent with sequence analysis, which suggests that the collagen molecule has features that can be related separately to intra- and intermicrofibrillar contacts (7).

Trelstad et al. (34) have proposed a similar model of fibril formation based on electron optical studies in which an intermediate formed from monomer assembles to form first thin and then thicker fibrils. The structure of the intermediate is not known but it could be a short microfibril or small bundle of short microfibrils (34).
How in vitro results such as we report here are related to in vivo assembly is not immediately clear. In vitro, the collagen solution is abruptly changed from nonaggregating to aggregating conditions. If the initial assembly of monomer is rapid and efficient, which is consistent with a large affinity constant or an early irreversible step, small aggregates could result with rapid depletion of monomer. Assembly of intermediates to fibrils might then be a slower less efficient process which would also require annealing. This kind of scheme could explain the polymorphic aggregates and loosely packed native fibrils seen in vitro as structures that have been kinetically trapped and perhaps fixed by covalent cross-linking in a normally unfavorable form. In vivo, there is presumably a great deal more control over assembly.

REFERENCES
Collagen fibril formation. Optimal in vitro conditions and preliminary kinetic results.

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