Does the Triple Helical Domain of Type I Collagen Encode Molecular Recognition and Fiber Assembly while Telopeptides Serve as Catalytic Domains?

EFFECT OF PROTEOLYTIC CLEAVAGE ON FIBRILLOGENESIS AND ON COLLAGEN-COLLAGEN INTERACTION IN FIBERS*

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Over the last several decades, it has been established that proteolytic removal of short, non-helical terminal peptides (telopeptides) from type I collagen significantly alters the kinetics of in vitro fibrillogenesis. However, it has also been observed that the protein is still capable of forming fibers even after complete removal of telopeptides. This study focuses on the characterization of this fibrillogenesis competency of collagen. We have combined traditional kinetic and thermodynamic assays of fibrillogenesis efficacy with direct measurements of interaction between collagen molecules in fibers by osmotic stress and x-ray diffraction. We found that telopeptide cleavage by pepsin or by up to 20 h of Pronase treatment altered fiber assembly kinetics, but the same fraction of the protein still assembled into fibers. Small-angle x-ray diffraction showed that these fibers have normal, native-like D-stagger. Force measurements indicated that collagen-collagen interactions in fibers were not affected by either pepsin or Pronase treatment. In contrast, prolonged (>20 h) Pronase treatment resulted in cleavage of the triple helical domain as indicated by SDS-polyacrylamide gel electrophoresis. The triple-helix cleavage correlated with the observed decrease in the fraction of protein capable of forming fibers and with the measured loss of attraction between helices in fibers. These data suggest that telopeptides play a catalytic role, whereas the information necessary for proper molecular recognition and fiber assembly is encoded in the triple helical domain of collagen.

Type I collagen is a major component of the extracellular matrix in all higher vertebrates, e.g. it is the main structural protein of skin, bone, and tendon (see, for example, Refs. 1 and 2). Each molecule is a heterotrimer composed of two α(I) chains and one α2(I) chain. It contains a long triple helical domain (~1000 residues from each chain) and short, non-helical terminal peptides (telopeptides).

In vitro, under appropriate conditions, type I molecules spontaneously form fibers that are virtually indistinguishable from native fibers by electron microscopy (see, for example, Ref. 3).

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The abbreviations used are: AcCol, acid-soluble collagen; PepCol, pepsin-treated collagen; PronCol, Pronase-treated collagen; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol.
Effect of Proteolytic Cleavage on Fibrillogenesis

AcCol, PepCol, and PronCol were purified by three cycles of salt precipitation and acidic acid resolubilization (23) and stored in 0.5 M acetic acid at 4 °C. Samples from each preparation were characterized by SDS-polyacrylamide gel electrophoresis (PAGE) (3% stacking gel and 6% separating gel, stained with Coomassie Blue R-250). Collagen concentration in solutions was measured by Sircol assay (Accurate Chemical & Scientific Corp.) and/or by optical absorbance in the 215–230 nm region. Both assays were calibrated using a set of standard collagen solutions of different concentrations.

Tyrosine content of collagen was estimated from the optical absorbance at 275.5 nm of collagen denatured in 6 M guanidine hydrochloride following the protocol proposed in Ref. 24. For type I collagen, it was shown previously that this spectroscopic method agrees with direct amino acid analysis within ~10% (25, 26).

Fibrillogenesis—The kinetics of fiber formation and equilibrium solubility of collagen at 32 °C were measured as described (22). Briefly, collagen was dialyzed against 2 mM HCl (pH 2.7). Aliquots from dialyzed collagen solutions (0.5–1.5 mg/ml) were mixed 1:1 on ice with 2× initiation buffer (200 mM sodium phosphate and 0.26 M NaCl with pH adjusted to give pH 7.4 in the mixture). The mixture was degassed for 5 min under vacuum and immediately placed into a Jasco V 560 spectrophotometer, where it was maintained at 32 °C. Fibrillogenesis kinetics was monitored by recording the optical density at 450 nm as a function of time. When no further change in the optical density was detected, the precipitate of assembled collagen fibers was spun down for 5 min at 14,000 g. Collagen solubility at 32 °C or the percentage of collagen competent to form fibers was evaluated from the protein concentration in the supernatant. The supernatant and precipitate were characterized by SDS-PAGE.

Bovin collagen was rearrested by slow concentration of AcCol, PepCol, and PronCol in 0.5 M acetic acid. Each solution was dialyzed at 4 °C in a Pierce Model 500 microdiaryzer system until a solid protein film was formed. Dialysis was performed against 50% polyethylene glycol (PEG; average M, 8000; U.S. Biochemical Corp.) solution in 0.5 M acetic acid. The film was further equilibrated in 40–50 weight % solution of PEG 8000 in 10 mM Tris and 2 mM EDTA (pH 7.5) for 2–3 days at 4 °C, washed in the same buffer to remove PEG, and air-dried at 4 °C. It was shown previously that such reconstituted films, prepared either from AcCol or from PepCol, consist of densely packed, native-like collagen fibers, whereas water inside fibers freely exchanges with surrounding solution (20, 21, 29). As a result, PEG osmotically compresses fibers. This action of PEG is counteracted by interaction between collagen fibers in fibers that is responsible for fiber swelling. From thermodynamic analysis of this force balance, it was shown that the corresponding force between helices (f) per unit of their length is given by (18) $f = \frac{F_{\text{PEG}} - d - \Delta\varepsilon}{\pi \eta / \lambda}$, provided that lateral packing of collagen can be approximated by a hexagonal lattice and interactions between collagen helices are pairwise additive.

Small-angle X-ray Diffraction—After force measurement, at least three samples of each collagen were rearrested in 6% PEG in 10 mM Tris and 2 mM EDTA (pH 7.5) and sealed in the same x-ray cell as for force measurement. The samples were exposed overnight in an Elliot GX-13 x-ray diffractometer equipped with a 100-μm focusing cup, a monochromator, and a multilayer x-ray lens (45-cm focal length; Osmic), and a single set of slits placed immediately after the lens. Diffraction patterns were captured on CRST-VN image plates (Fuji) and read using a BAS2500 image plate scanner (Fuji). The sample-to-plate distance was ~40 cm. The size of the x-ray beam at the focal spot on the plate was ~200 × 320 μm. The resolution was estimated as $2d_{\text{int}} = 0.063 \AA^{-1}$. The lowest measurable scattering vector $q = 0.04–0.08 \AA^{-1}$. This could not be improved due to limitations associated with the design of the x-ray lens. However, such resolution was sufficient for the purpose of this work.

RESULTS

Enzymatic Treatment of Collagen—We evaluated integrity of collagen molecules by SDS-PAGE and by estimating the number of tyrosine residues/molecule from UV absorption spectra. We assumed that rat tail tendon collagen is similar to mouse type I collagen, whose complete primary sequence is known (29, 30). Based on the mouse sequence (α1(1) chain: CA11_MOUSE, Swiss-Prot accession number P11087; and α2(1) chain: CA21_MOUSE, Swiss-Prot accession number Q01149), one expects an intact molecule to contain 14 tyrosines: 12 in telopeptide chains and 2 in the triple helical region of the α2(1) chain.

Judging from SDS-PAGE, AcCol that did not undergo any enzymatic treatment contained α1(1) and α2(1) chains along with higher molecular weight complexes (Fig. 1, lane 1). The complexes are due to a variety of factors: 1) cross-links between telopeptide chains of the same molecule or between a telopeptide on one molecule and a helical domain on another molecule (9, 10). From UV absorption at 275 nm, the number of tyrosine residues in AcCol was estimated as ~10 (Table 1). Apparently, some AcCol molecules had damaged telopeptides.

The pepsin treatment of collagen removed higher molecular weight complexes and reduced the molecular weight of α1(1) and α2(1) chains (Fig. 1, compare lanes 1 and 2). The observed decrease in the number of tyrosine residues/molecule from ~10 in AcCol to ~5 in PepCol supports this interpretation, as previously shown (26, 29). After 20 h of Pronase treatment (20-h PronCol), the major α1 and α2 bands on SDS-PAGE were similar to those in PepCol (Fig. 1, compare lanes 2 and 9). From UV absorption, we found ~2 tyrosines/20-h PronCol molecule. Most likely, these are the tyrosine residues located within the triple helical domain of the α2 chain. Apparently, all telopeptide tyrosines are removed by 20 h of Pronase treatment, including those at the interface between the triple helix and carboxyl-terminal telopeptides. This indicates complete telopeptide digestion, in agreement with previous reports (12, 13, 32).

In contrast to pepsin, Pronase treatment led to two addi-
Genetically various collagen preparations are shown in Figs. 1 and 2. Lanes 1 and 2 show the composition of fibrillogenesis-incompetent fractions from these preparations. Each fraction was separated by centrifugation (as supernatant) after the end of the measurement of fibrillogenesis kinetics at 32 °C (see Fig. 2a). No protein was observed in the supernatant after fibrillogenesis of AcCol and PepCol. Hence, the corresponding empty lanes are not shown. A small shift of the α1 and α2 bands in PepCol and PronCol (lanes 2 and 3) with respect to similar bands in AcCol (lane 1) is a result of telopeptide cleavage. Additional α1', α1'', α2', and α2'' bands appearing in lanes 3–8 are products of the cleavage of the triple helical domain of collagen.

**Collagen Estimated average No. of Tyr residues/molecule**

| Collagen   | Estimated average No. of Tyr residues/molecule |
|------------|-----------------------------------------------|
| AcCol      | 10 ± 2                                        |
| PepCol     | 5 ± 1                                         |
| 20-h PronCol | 2.3 ± 0.5                                    |
| 44-h PronCol | <1.6                                         |
| 68-h PronCol | <1.2                                         |

*The values were obtained using a 1500 m–1 cm–1 extinction coefficient for Tyr at 275.5 nm, as described (24). For type I collagen, this spectroscopic method agrees with direct amino acid analysis within ~10% (25, 26). In our case, the most likely source of error was base-line subtraction. For AcCol, PepCol, and 20-h PronCol, the uncertainty in base-line subtraction did not exceed 20%. For 44- and 68-h PronCol, the base-line subtraction was unreliable, and only upper bounds for the tyrosine content could be estimated.*

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### Collagen Packing in Reconstituted Films—To assess changes in structure and interaction between collagen molecules in fibers, we prepared reconstituted protein films as described under “Materials and Methods.” The structure of these films can be determined by comparing their small-angle x-ray diffraction patterns with a similar pattern from native rat tail tendons, as shown in Fig. 3.

In native tendon fibers, a characteristic set of multiple reflections can be clearly seen (Fig. 3, curve a). These reflections are higher orders of diffraction arising from d = 670-Å axial periodicity in collagen fibers. The positions of maxima in the q-space are given by q(n) = 2πnd, where n is the order of the diffraction and q is the scattering vector. Note that the 5th, 9th, 12th, 20th, and 21st orders are significantly stronger than their neighboring peaks. This pattern is a signature of the native D-stagger of collagen fibers (4, 6, 7, 33).

Long Pronase digestion (44 and 68 h) resulted in extremely slow fibrillogenesis kinetics so that the process was not complete after 24 h. Thus, although the soluble protein fraction contained primarily α1', α1'', α2', and α2'' chains, it also had some intact α1 and α2 chains (Fig. 1, lanes 6 and 8).

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The small-angle diffraction pattern from reconstituted AcCol films (Fig. 3, curve b) is very similar to the pattern from native fibers in terms of both positions and relative intensities of the diffraction peaks, consistent with what was reported previously (4, 7). However, the ordering of collagen molecules in reconsti-
Fig. 2. a, fibrillogenesis kinetics measured by light absorbance. Curve 1, 0.35 mg/ml AcCol; curve 2, 0.25 mg/ml PepCol; curve 3, 0.25 mg/ml 20-h PronCol; curve 4, 0.5 mg/ml 44-h PronCol; curve 5, 0.8 mg/ml 68-h PronCol. The inset shows initial stages of the process up to 60 min. The optical density is normalized to give the ratio of collagen in the precipitate to total collagen after the end of each kinetic experiment. (The absolute value of the optical density is poorly reproducible and may be misleading since it depends on the size of forming aggregates.) b, effect of the length of Pronase treatment on the fraction of collagen that forms fibers at 32°C. Each point corresponds to the ratio of collagen in the precipitate to total collagen measured after the end of the corresponding kinetic experiment shown in a. The error bars represent estimated errors of collagen concentration measurements (see “Materials and Methods”).

Fig. 3. Small-angle x-ray diffraction patterns from native collagen fibers (curve a), reconstituted AcCol fibers (curve b), PepCol (curve c), 20-h PronCol (curve d), 44-h PronCol (curve e), and 68-h PronCol (curve f). For clarity, the patterns are shown after subtraction of background scattering, fitted separately for each sample by a stretched exponential curve. No data filtering or noise reduction procedures were used. The number of the diffraction order corresponding to each relatively strong peak is shown at the top of the graph.

The net force between collagen helices is plotted versus average interaxial separation ($d_{int}$) in Fig. 4 for native and reconstituted fibers. Note that native tendon fibers contain collagen and other tissue-specific components. We found that an overnight wash in 0.1 M phosphate and 2 M glycerol (pH 7.5) resulted in the loss of tissue-specific crystal-like lateral packing of collagen (observed in tendons from some (but not all) species and not observed in skin and other tissues (5, 6)). The collagen-collagen forces in washed native fibers were identical to forces in reconstituted AcCol, PepCol, and 20-h PronCol fibers (Fig. 4a).

We found previously that the net force between collagen helices in fibers is a sum of repulsive and attractive interactions (20, 21). The balance between the repulsion and attraction determines the equilibrium separation between molecules. The repulsion dominates at shorter and the attraction dominates at larger interaxial separations. The attraction is responsible for molecular recognition between collagen helices and for their spontaneous assembly from solution into native-like fibers. The repulsion is shown in Fig. 4b by the dashed straight line. The attraction leads to a downward deviation of the net force curve from this straight line. The attraction increases with increasing temperature (Fig. 4b), resulting in temperature-favored fiber assembly.

Note that the helix-helix forces in washed native fibers, in reconstituted PepCol fibers, and in reconstituted 20-h PronCol fibers were virtually identical at all temperatures (Fig. 4b). Even complete telopeptide removal had no visible effect on helix-helix interaction.

Longer Pronase treatment (44 and 68 h) strongly suppressed the attractive component of the net force so that the downward deviation from pure helix-helix repulsion was much weaker
To verify that this is the suppression of the attractive component rather than an enhancement of the repulsive component of the net force, we used glycerol, which is a specific attraction inhibitor (22). We found that 2 M glycerol reduced the attraction between PepCol helices, but it had almost no effect on interaction between 44-h PronCol helices (Fig. 5b). In other words, the observed collagen-collagen attraction is sensitive to cleavage of the triple helical domain, but not to telopeptide removal.

DISCUSSION

Effect of Telopeptides on Kinetics of Fiber Nucleation and Growth—Several mechanisms of collagen fibrillogenesis that address the role of different parts of the collagen molecule and the effect of the environment (pH, ionic strength, specific ions, and various additives) were proposed in the literature (see, for example, Ref. 8). It is now commonly accepted that fibrillogenesis involves at least two distinct steps: nucleation and fiber growth. It was suggested that fibril nuclei may exist as metastable intermediates that have a long life-time at low temperature. By analyzing fibrillogenesis kinetics during heating/cooling cycles, it was shown that collagen solution has a long “thermal memory.” Repeated heating results in fibrillogenesis with a considerably shorter lag time. Proteolytic cleavage of telopeptides abolishes the thermal memory (34). It also leads to an increase in the fibrillogenesis nucleation time (11, 16). The larger the fraction of the amino-terminal telopeptides that is removed, the longer the nucleation delay (12) (see also Fig. 2a). Hence, it was postulated that amino-terminal telopeptides accelerate fiber nucleation.

Evidence was also reported that removal of carboxyl-terminal telopeptides from collagen slows down fibril growth (12). Consistently, synthetic peptides containing amino acids found in carboxyl-terminal telopeptides were shown to inhibit fibril assembly when added to a solution of intact collagen, apparently by competing with carboxyl-terminal telopeptides for binding sites on collagen (17). Thus, it was suggested that carboxyl-terminal telopeptides are essential for the fibril growth step.

Effect of Telopeptides on Fiber-forming Competency of Collagen, on Fiber Structure, and on Fiber Stability—In this work, we found that even complete removal of telopeptides by 20 h of Pronase treatment had no significant effect on the fiber-forming competency of collagen. Although fibrillogenesis kinetics was altered (Fig. 2a), in agreement with previous reports, virtually all molecules with intact triple helical domains assembled into fibers as if the telopeptides were present (Figs. 1 and 2b). Only those molecules whose triple helical domains were cleaved by 44 or 68 h of Pronase treatment lost their ability to assemble into fibers.

Small-angle x-ray diffraction showed that inside reconstituted collagen films, AcCol, PepCol, and 20-h PronCol molecules organized into fibers whose D-staggered packing was identical to that of native fibers (Fig. 3). The films were reconstituted under acidic conditions (see “Methods and Methods”) that suppress collagen-collagen recognition. To ensure their stability, the films had to be equilibrated at neutral pH under osmotic stress for at least several days. Apparently, collagen slowly reorganizes into native-like fibers during this equilibra-
Catalytic Versus Recognition and Structural Role of Telopeptides—Thus, measurements of collagen solubility after kinetic PronCol) (Fig. 5).

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Thus, measurements of collagen solubility after kinetic data suggest that telopeptides are not essential for the ability of collagen to form native-like, D-staggered fibers. The information that is essential for noncovalent interactions holding fibers together appears to be encoded in the triple helical domain of the protein. In contrast, covalent interactions, which are responsible for fiber stability with respect to severe stress, involve telopeptides (see, for example, Refs. 2, 9, and 10).

This interpretation may seem to be at odds with previous reports that AcCol, PepCol, and 20-h PronCol fibers have different morphologies when studied by electron microscopy (11, 13–16). However, staining by heavy metal ions used in electron microscopy strongly affects fiber structure, as shown by x-ray diffraction from native rat tail tendons (35). Drying, high vacuum, and high energy electron beam impose additional stresses. It is possible that only AcCol fibers, stabilized by intermolecular covalent cross-links, are able to retain their basic structure under such conditions.

Note that previously reported (11, 12, 16, 34) and our own (Fig. 2a) kinetic data suggest that telopeptides accelerate collagen fiber nucleation. However, the exact nature of the nuclei and the mechanism of telopeptide effect on their formation remain unknown. It was shown that telopeptides bind to specific sites on collagen molecules (12, 17), but the exact role of this binding in fibrillogenesis is unclear. We can only speculate that binding of floppy, non-helical peptides may occur faster than proper alignment of long, rigid molecules. The binding may be transient, and/or it may be energetically insignificant compared with the measured interaction (Figs. 4 and 5) between triple helical domains. Still, it may accelerate fibrillogenesis by placing the molecules in correct register with each other (36).

In other words, some questions remain unresolved. However, regardless of what their exact role is, telopeptides appear to perform a catalytic function rather than a recognition or an energetic function. In our opinion, this hypothesis gives the most consistent explanation to the existing experimental data.

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