Unhydroxylated Triple Helical Collagen I Produced in Transgenic Plants Provides New Clues on the Role of Hydroxyproline in Collagen Folding and Fibril Formation

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Stéphanie Perret‡§, Christine Merle§, Simonetta Bernocco‡§, Patricia Berland§, Robert Garrone‡, David J. S. Hulmes‡, Manfred Theisen§, and Florence Ruggiero‡**

From the ‡Institut de Biologie et Chimie des Protéines, CNRS UMR 5086, Université Claude Bernard Lyon I, 7 Passage du Vercors, F-69367 Lyon Cedex 07 and §Meristem Therapeutics, 8 Rue des Frères Lumière, 63100 Clermont-Ferrand, France

Human unhydroxylated homotrimeric triple-helical collagen I produced in transgenic plants was used as an experimental model to provide insights into the role of hydroxyproline in molecular folding and fibril formation. By using chemically cross-linked molecules, we show here that the absence of hydroxyproline residues does not prevent correct folding of the recombinant collagen although it markedly slows down the propagation rate compared with bovine fully hydroxylated homotrimeric collagen I. Relatively slow cis-trans-isomerization in the absence of hydroxyproline likely represents the rate-limiting factor in the propagation of the unhydroxylated collagen helix. Because of the lack of hydroxylation, recombinant collagen molecules showed increased flexibility as well as a reduced melting temperature compared with native homotrimers and heterotrimers, whereas the distribution of charged amino acids was unchanged. However, unlike with bovine collagen I, the recombinant collagen did not self-assemble into banded fibrils in physiological ionic strength buffer at 20 °C. Striated fibrils were only obtained with low ionic strength buffer. We propose that, under physiological ionic strength conditions, the hydroxyl groups in the native molecule retain water more efficiently thus favoring correct fibril formation. The importance of hydroxyproline in collagen self-assembly suggested by others from the crystal structures of collagen model peptides is thus confirmed experimentally on the entire collagen molecule.

Collagen I is a fibrillar collagen, found in bone, skin, tendon, and several other vertebrate connective tissues, which is among the most abundant structural proteins of the extracellular matrix. Two different forms of the triple-helical molecule may occur, depending on the presence of one or both of the genetically distinct chains α1(I) and α2(I). The most studied and widely distributed form is the heterotrimer [α1(I)2α2(I)], although significant amounts of the homotrimer [α1(I)]3 are present in embryonic and fetal tissues (1). The sequence of the large triple-helical domain consists of a repeated tripeptide sequence Gly-X-Y, where X and Y are frequently proline and hydroxyproline, respectively (2). The strict recurrence of this sequence ensures the correct folding of three α chains into a stable triple helix extending over a thousand residues, whereas the high content of hydroxyproline increases the thermal stability of the molecule.

Fibril formation is considered as a self-assembly process that depends on the intrinsic properties of the molecules themselves such as the distribution of polar and hydrophobic residues, the persistence of the short non-helical segments at the extremities of the mature molecule (3, 4), and the possible copolymerization into a single fibril of several different fibrillar collagen types. Different α chain stoichiometries are also an important factor. For instance, the collagen I homotrimer can form fibrils in vitro although less efficiently than the heterotrimer (5). The most obvious difference between the two molecules is the absence of the α2(I) chain in the homotrimer that is present in the heterotrimer. This prompted the speculation that chain composition can dictate fibril formation kinetics by differences in entropy. Whereas cells might provide additional control of the formation and deposition of fibrils in vivo, in vitro reconstitution of fibrils is triggered simply by warming, neutralizing the pH, and increasing the ionic strength to physiological values.

Although fibril formation appears to be a very simple process, the molecular mechanisms that drive self-assembly of monomers are far from elucidated. The general thermodynamic mechanism commonly accepted is that fibril formation is an entropy-driven process accompanied by a loss of water from the surface of the molecules (6). The importance in fibril formation of structured water molecules at the surface of collagen monomers was recently brought out by studying the crystal structures of collagen model peptides (7–13). The resolution of these triple-helical peptide structures, in particular (Pro-Pro-Gly)10 (9, 12), (Pro-Hyp-Gly)4-Pro-Hyp-Ala-(Pro-Hyp-Gly)5 (7, 8), and (Pro-Hyp-Gly)15 (13), revealed that whereas the overall molecular conformations are similar, different crystal packing arrangements are observed. Analysis of hydration patterns in the presence or absence of hydroxyproline residues provides an unexpected and novel clue to the understanding of the collagen self-assembly mechanism. From these structural data, it was thus speculated that hydroxyproline residues may have a critical role in the organization of the water network surrounding the triple helix resulting in a lateral molecular packing similar to that which occurs in fibrils (9). Indeed, as early as in the 1950s, the role of hydroxyproline residues in the stabilization of the collagen fibril structure through hydrogen bonds has been highlighted (14).
The study of the role of hydroxyproline in fibril formation by routine in vitro fibril formation experiments suffers from difficulties in producing large amounts of stable unhydroxylated collagen I molecules. Recently, we have shown (15) that tobacco plants provide a useful expression system for the large scale production of recombinant collagen. Interestingly, although hydroxylation of proline residues did not occur in plants, the recombinant collagen adopted a stable triple-helical conformation, and thus this provides an impetus for studying the role of hydroxyproline in fibril formation. For this purpose, we have used this system to prepare adequate amounts of unhydroxylated recombinant collagen I homotrimer to study both its molecular characteristics and its ability to form fibrils. The results indicate that although the molecule shows features similar to the native collagen I homotrimer and heterotrimer, the experimental conditions required for fibril formation differ markedly from those previously established for native collagen I. The importance of hydroxyproline in self-assembly as proposed from the crystal structures of collagen model peptides is thus here experimentally confirmed on the whole collagen molecule.

MATERIALS AND METHODS

Collagen Purification—Collagens were extracted from fetal calf bones by pepsin digestion and separated by repeated salt fractionation as described previously (16). The 0.7 M NaCl and 0.9 M NaCl precipitates containing the [α1(I)]2ε2(I) collagen I heterotrimer (Het I)1 and the [α1(I)]1, collagen I homotrimer (Hom I), respectively, were analyzed by 6% SDS-PAGE. Collagen I different isoforms were then dissolved in 0.5 M acetic acid, dialyzed against 0.1 M acetic acid to eliminate salt, and then lyophilized.

Recombinant collagen was extracted and purified from field-grown tobacco plants transformed with human pro-α1(I) chain cDNA lacking the N-propeptide coding sequence (15). Fresh tobacco leaves and stems were frozen and homogenized in liquid nitrogen. After extraction in 0.5 M acetic acid containing 0.2 M NaCl, the recombinant collagen was purified by repeated salt fractionation. The 0.9 M precipitate containing the recombinant collagen (rColl I) was solubilized in 0.1 M acetic acid and purified by CM-Sepharose chromatography in 25 mM sodium acetate, pH 4.8. The purified recombinant collagen was then dialyzed against 0.1 M acetic acid and lyophilized.

Gel and Western Blot Analysis—Samples were analyzed by either 6, 5–15, or 3.5–5% gradient SDS-polyacrylamide gel electrophoresis (PAGE) as indicated. Gels were then stained with Coomassie Blue or electrotransferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore) in 10 mM CAPS, pH 11, 5% methanol, overnight. After saturation, membranes were probed with polyclonal antibodies against human collagen I (Novotec, France) followed by incubation with secondary antibodies conjugated to alkaline phosphatase. Immunoblots were revealed with the alkaline phosphatase conjugate substrate kit (Bio-Rad).

Intramolecular Cross-linking of Collagen Molecules—Collagen solutions were prepared at 500 μg/ml in 0.1 M acetic acid. Solutions were diluted in an equivalent volume of 2X PBS, and the pH was adjusted to 7.0 with 1 M NaOH. The N-hydroxysuccinimide ester cross-linker disuccinimidyl glutarate (DSG, Pierce) was dissolved in MeSO and subsequently added to the collagen solutions at a ratio 1:50. Cross-linking reactions were allowed to proceed for 15 min at 4 °C and were quenched by the addition of a large excess of Tris. The efficiency of the collagen molecule cross-linking was analyzed by 3.5–5% gradient SDS-PAGE. Cross-linked collagen molecules were then dialyzed against 0.1 M acetic acid at 4 °C before circular dichroism experiments.

Circular Dichroism—Solutions of collagen or cross-linked collagen molecules were dissolved in 0.1 M acetic acid and denatured at 80 °C for 15 min immediately before CD analysis. Renaturation CD spectra were recorded at 222 nm at 10 °C on a CD6 Jobin Yvon spectropolarimeter equipped with a 1-mm path length thermostatically controlled quartz cuvette. For comparison, spectra were normalized, and the fraction of folded molecules was calculated as follows: F = (θobs − θmin)/(θmax − θmin), where θobs represents the observed molar ellipticity; θmin was measured before denaturation at 10 °C, and θmax corresponds to the value measured after denaturation at 60 °C. To compare the data from the different samples, the time (τ222) at which F = 0.5 was determined as stated above.

Electron Microscopy and Fibril Diameter Measurement—After fibril and SLS crystallite formation, drops of the different samples were placed onto Formvar-carbon grids and then negatively stained with 2% phosphotungstic acid at pH 7.4 and/or positively stained with 0.2% phosphotungstic acid and 1% uranyl acetate as indicated. Stained aggregates were examined using a Philips CM 120 transmission electron microscope at the “Centre de Microscopie Appliquée à la Biologie et à la Géologie” (Université Claude Bernard, Villeurbanne, France). Fibril diameters were measured directly from calibrated electron micrographs, and the values were plotted as histograms.

In Vitro Fibrillogenesis Kinetics—Chilled quartz cuvettes were filled with collagen solutions (Coll I, Hom I, and Het I) at a final concentration of 200 μg/ml against different buffers at 4 °C, overnight. The following buffers were tested: phosphate-buffered saline, pH 7.4 (PBS, 8 mM Na2HPO4, 0.15 mM KH2PO4, 2.6 mM KCl, 0.137 mM NaCl), 20 mM KH2PO4, 140 mM NaCl, pH 7.4 and 9.0; 20 mM KH2PO4, 1 mM NaCl, pH 7.4; 10 mM KH2PO4, pH 7.

SLS crystals were analyzed by dialyzing collagen monomers against 0.4% ATP in 0.1 M acetic acid for 72 h at 4 °C.

RESULTS

Purification of Collagen—Collagen I heterotrimer and homotrimer were extracted from embryonic calf bones by pepsin digestion and then isolated by repeated differential salt precipitation. Both heterotrimeric and homotrimeric forms of collagen I are present in calf bones, as shown previously (15), and both isoforms were used for this study. The predominant form of collagen I, the heterotrimer [α1(I)]2ε2(I), referred to as Het I, precipitated selectively at 0.7 M NaCl, whereas the homotrimer [α1(I)]1, referred to as Hom I, was recovered in the 0.9 M NaCl precipitate (Fig. 1A, lane 2, and 3). To include the possibility that the different isoforms purified by salt fractionation contained collagen III, we examined the electrophoretic behavior of these molecules under reducing or non-reducing conditions. No bands migrating in the position of trimers were observed in the absence of reducing agent, indicating that no collagen III was present in the samples (Fig. 1B). The absence of contaminating

1 The abbreviations used are: Het I, [α1(I)]2ε2(I) bovine collagen I heterotrimer; Hom I, [α1(I)]1 bovine collagen I homotrimer; rColl I, recombinant human collagen I; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; CAPS, 3-(cyclohexylamino)propanesulfonic acid; DSG, disuccinimidyl glutarate; SLS, segment-long spacing.
heterotrimers in the collagen I homotrimer sample was attested by Western blot analysis using polyclonal antibodies against bovine collagen I. No band migrating at the α2(I) position was detected in the homotrimer sample, whereas this band was clearly present in the 0.7 M precipitate (Fig. 1C).

Recombinant homotrimeric collagen I (rColl I) from transgenic tobacco plants that carry the construct lacking the N-propeptide domain was extracted by acetic acid and purified by differential salt precipitation as described (15). It was shown previously that the C-propeptides were cleaved during the extraction procedure and thus the purified molecules correspond to mature collagen. However, as observed previously, rColl I migrated faster than the α1(I) bovine collagen chain (Fig. 1A, lane 4). This migration was shown to be due to the lack of hydroxylation in the plant system. The purity of the different collagen samples was judged by Coomassie Blue staining of the 5–15% gradient SDS-PAGE (Fig. 1) and by amino acid composition analysis (not shown).

Specific Properties of Unhydroxylated Collagen Molecules—Recombinant collagen is folded into a triple helix similar to that of native collagen I (15), as judged by circular dichroism, proteinase resistance, and electron microscopy after rotary shadowing. Because of the lack of prolyl hydroxylation however, the melting temperature (Tm) of the unhydroxylated molecule is relatively low (30°C). To investigate the consequences of such properties unique to unhydroxylated trimers, native and recombinant collagens were compared in terms of stability, flexibility, and ability to form fibrils.

It has been shown using short host-guest peptides that the presence of hydroxyproline accelerates refolding of the collagen triple helix (17). The availability of significant amounts of both hydroxylated and non-hydroxylated trimers allowed this conclusion to be tested on full-length collagen molecules. Because studies on collagen renaturation are hampered by problems of chain misalignment, we chose to introduce intramolecular cross-links, as present naturally in e.g., collagen III, before denaturation-renaturation. In parallel, homotrimeric bovine collagen I was cross-linked as a control. An N-hydroxysuccinimide ester cross-linker presenting a short spacer arm of 7.7 Å (DSG) was used to favor intra-molecular cross-links and avoid the formation of higher molecular weight aggregates. As expected, cross-linked trimers were predominantly observed on a 3.5–5% electrophoretic gel, whereas no monomer chains were present, and only a faint band migrating at the top of the gel, corresponding to supra-molecular aggregates, was detected (Fig. 2A). At 10°C, spectra obtained by circular dichroism for the recombinant cross-linked collagen were typical of a triple-helical conformation. Cross-linking had no effect on the Tm of the recombinant collagen (30°C; Fig. 2B), indicating no change in overall thermal stability. Following denaturation, however, hydroxylated native homotrimers (Hom I) renatured more quickly than unhydroxylated recombinant homotrimers (rColl I; Fig. 2C). Although triple helix formation is not prevented by the lack of prolyl hydroxylation, correct prolyl hydroxylation favors helix assembly.

The flexibility of rColl I molecules, another parameter that might affect fibril formation, was assessed by dynamic light scattering at different temperatures (Fig. 3). At 11°C, the diffusion coefficient of the native heterotrimer (Het I) was as expected (6.6 ± 1.6 × 10⁻⁹ cm² s⁻¹) (18). The diffusion coefficient of the native homotrimer (Hom I) was somewhat greater, whereas that of the recombinant homotrimer (rColl I) was greater still. This indicates that the recombinant molecule adopts a more compact and therefore more flexible conformation than the native collagens. At 20°C, the temperature used for fibril formation (see below), the diffusion coefficients of all three collagens increased, consistent with a general increase in compactness/ flexibility. This trend continued at 25 and 35°C, although because of the relatively low Tm, measurements at 35°C were not carried out for recombinant collagen. It is noteworthy that the diffusion coefficients for the recombinant homotrimer at 20 and 25°C were similar to that for the native heterotrimer at 35°C.

The capacity of the recombinant collagen to form supramolecular structures was studied using SLS crystallites, an artificial mode of molecular packing in which polymers such
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Critical conditions allowing formation of striated fibrils from unhydroxylated recombinant collagen were identified by varying the ionic strength of the fibrillogenesis buffer. Under all other conditions (variations of pH and temperature), no striated fibrils were formed. Only when recombinant collagen was incubated in low ionic strength buffer (10 mM phosphate, pH 7) was striated fibril assembly observed. Under these conditions, cross-banded fibrils were predominant, and the measured periodicity was 65.9 ± 0.8 nm which is close to the 67 nm expected value (Fig. 6, A–C). Under the same conditions, Het I formed a meshwork of long and thin striated fibrils (Fig. 6, D and E), whereas Hom I only aggregated into non-banded fibrils (Fig. 6F). The rColl I fibrils were particularly heterogeneous in size and morphology (Fig. 6, A–C). Fibril diameter measurements confirmed that recombinant unhydroxylated fibrils presented a broad distribution with a minimum at 15 nm and a maximum at about 500 nm. In contrast, Het I showed the highest frequency of striated fibrils with a diameter of about 40 nm (Fig. 6G).

Fibril formation in 10 mM phosphate was followed by monitoring turbidity at 313 nm as a function of time. The amplitude of the plateau value is a function of both the amount of reconstituted fibrils and their final width. Fibrillogenesis kinetics under these conditions (10 °C, 10 mM phosphate, pH 7) were instantaneous whichever collagen was used. No significant difference was observed between the kinetics for Het I and rColl I (Fig. 7). The morphology of the fibrils obtained after completion of turbidity measurements was identical to that previously shown (Fig. 6). Thus, the formation of striated fibrils with unhydroxylated collagen, contrary to hydroxylated homotrimeric collagen I, is dependent on the low ionic strength of the buffer. As shown for native collagens, fibril formation increased substantially the melting temperature of the recombinant collagen which reached 36 °C instead of 30 °C for the isolated molecules (data not shown).

DISCUSSION

The study of collagen molecules that are correctly folded but lack hydroxyproline residues, as produced in transgenic plants, provides clues to understand how these residues contribute to triple-helical folding and, more strikingly, useful insights into the molecular mechanisms that drive fibrillogenesis.

Role of Hydroxyproline in Collagen Folding—Hydroxyproline plays an unquestionable role in the thermal stability of collagen molecules, where the number of hydroxyproline residues in the triple-helical domains is directly related to the melting temperature. In collagen-like peptides that form triple helices, the substitution of hydroxyproline for proline in the Y-position of the repeating Gly-X-Y triplets increases the thermal stability by as much 15 °C (19, 20). As a result, the melting temperatures of recombinant unhydroxylated collagen I produced in plants and of fully hydroxylated parental collagen I homotrimer show distinct disparities (30 °C for the recombinant collagen versus 45 °C for the native bovine homotrimer). The role of hydroxyproline in collagen folding is less clear. When hydroxylation is blocked by addition of α,α′-dipyridyl, fibroblasts synthesize the unhydroxylated form of procollagen I, known as protocollagen (21). Surprisingly, the data show that the folding of protocollagen is more efficient than that of fully hydroxylated collagen. Apparently, because it lacks hydroxyproline, the stability of mismatched triple-helical regions is decreased, and thus the protein is less likely to be locked into unfavorable conformations that impede the propagation step. However, the folding of protocollagen I was found to be less efficient than the folding of collagen III. The latter formed fully aligned triple helices in vitro most likely because of the presence of interchain disulfide bonds within the C-terminal end of the triple-helical domain (22). To avoid possible α chain misalignments that could disturb the folding rate, we chemically cross-linked the collagen molecules before refolding the thermally denatured protein. Identical fractions of folded triple helices were reached with fully hydroxylated collagen I homotrimer and recombinant collagen indicating that, when cross-linked, the helix-to-coil transition of both collagens was reversible. However, we observed a marked difference in the folding rate that was 5-fold faster for native homotrimeric collagen than for unhydroxylated recombinant collagen.

The increased folding rate of collagen III in the presence of cis-trans-isomerase indicated that cis-trans-isomerization of proline/hydroxyproline is a rate-limiting factor in collagen molecular assembly (23). In related work, substitution of (4R)-
fluoroproline for (4R)-hydroxyproline in collagen-like peptides was shown to increase the Tm of the triple helix (24). Because (4R)-fluoroproline did not provide a site for hydrogen bonding of water, these authors proposed that an electron-withdrawing inductive effect of the hydroxyl or fluoro groups favors the trans-configuration of the peptide bond required for formation of the triple helix. As a consequence, the inductive effect of the hydroxyl group of hydroxyproline enhances the stability of the triple helix by favoring the requisite trans-conformation of the Hyp bond. In our results, the relatively slow cis-trans-isomerization in the absence of hydroxyproline thus likely became the rate-limiting factor for the propagation of the recombinant unhydroxylated collagen helix. In agreement with this concept, the use of collagen-like peptides for refolding studies showed that all triplets of the form Gly-Xaa-Hyp promoted rapid folding, whereas Gly-Xaa-Pro triplets were less favorable (17).

Role of Hydroxyproline in Fibril Assembly—Collagen self-assembly is an entropy-driven process that depends on ionic strength, pH, and temperature. Unlike hydroxylated heterotrimers or homotrimers, we found that unhydroxylated homotrimers did not assemble into striated fibrils under physiological buffer conditions. Instead, fibril formation of unhydroxylated collagen required both low ionic strength and temperature, the latter being imposed by the relatively low thermal stability of the molecule.

To investigate further how the lack of hydroxylation might affect physical properties, we showed by dynamic light scattering that unhydroxylated collagen molecules appeared to be more compact/flexible than native collagen at temperatures between 11 and 25 °C. At 25 °C, the compactness/flexibility of the recombinant molecule was found to be comparable with that of the native heterotrimer at 35 °C, i.e. in both cases below their respective melting temperatures. Increased flexibility might actually favor fibril formation because molecules are likely to adopt a bent conformation within fibrils (25–27). Thus the flexibility of the recombinant molecule at 20–25 °C might be conducive to fibril formation. However, our data showed that at 20 °C unhydroxylated molecules did not form striated fibrils at physiological pH and ionic strength. In contrast, hydroxylated homotrimers and heterotrimers did assemble under these conditions, despite their reduced flexibility. Thus differences in molecular flexibility do not appear to be a major influence on the ability to form striated fibrils.

Low ionic strength conditions appear therefore to be the major requirement for fibril formation of unhydroxylated col-

trans-config.
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The integrity of the triple helix is a necessary prerequisite for correct fibril formation, and the telopeptides are known to play a catalytic role (28). In our experiments recombinant collagen was obtained in mature form, i.e., after removal of propeptides but without the use of pepsin, thereby leaving the telopeptides mostly intact at the N terminus and with 3–5 residues remaining at the C terminus (data not shown). In contrast, both the bovine heterotrimer and homotrimer were isolated following pepsin extraction. All molecules were indistinguishable by analysis of SLS crystallites formed in vitro. The observation that both bovine heterotrimers and homotrimers formed striated fibrils under physiological buffer conditions, whereas recombinant homotrimers with more intact telopeptides did not, further reinforces our conclusion that the differences in fibril formation were due to the lack of hydroxylation.

Fibril formation increases the thermal stability of native bovine collagen by as much as 10 °C (29). We showed that the melting temperature of recombinant collagen fibrils was also increased by about 6 °C, showing that thermal stability is raised once the unhydroxylated molecules are stabilized by the fibril structure. This result might be of interest for its use as biomaterials. We conclude that hydroxyproline might contribute more than previously thought to correct fibril formation under experimental conditions mimicking those that can occur physiologically. Understanding the molecular mechanisms that drive collagen self-assembly must therefore take account of the role of hydroxyproline.

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