Recombinant Procollagen II: Deletion of D Period Segments Identifies Sequences That Are Required for Helix Stabilization and Generates a Temperature-sensitive N-Proteinase Cleavage Site*

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A cDNA cassette system was used to synthesize recombinant versions of procollagen II in which one of the four blocks of 234 amino acids that define a repeating D periods of the collagen triple helix were deleted. All the proteins were triple helical and all underwent a helix-coil transition between 25 and 42 °C as assayed by circular dichroism. However, the details of the melting curves varied. The procollagen lacking the D1 period unfolded 3 °C lower than a full-length molecule. With the procollagen lacking the D4 period, the first 25% of unfolding occurred at a lower temperature than the full-length molecule, but the rest of the structure unfolded at the same temperature. With the procollagen lacking the terminal D0.4 period, the protein unfolded 3 °C lower than the full-length molecule and a smaller fraction of the protein was secreted by stably transfected clones than with the other recombinant procollagens. The results confirmed previous suggestions that the collagen triple helix contains regions of varying stability and they demonstrated that the two D periods at the end of the molecule contain sequences that serve as clamps for folding and for stabilizing the triple helix. Reaction of the recombinant procollagens with procollagen N-proteinase indicated that in the procollagen lacking the sequences, the D1 period assumed an unusual temperature-sensitive conformation at 35 °C that allowed cleavage at an otherwise resistant Gly-Ala bond between residues 394 and 395 of the α1(II) chain.

The fibrillar collagens are major structural proteins that largely define the size, shape, and strength of tissues in most complex organisms (1–4). In addition, collagen fibrils are encountered in unusual biological situations such as the byssal threads whereby mussels adhere to solid surfaces (5) and in tube worms that live near deep sea hydrothermal vents (6). Collagen fibrils are formed by the spontaneous self-assembly of collagen monomers (1–4). The monomers of the major fibrillar collagens, types I, II and III, are very similar in structure in that they consist of 338 or 343 consecutive repeating tripeptide sequences of -Gly-Xxx-Yyy- tripeptide units. The -Xxx- position of the sequence is frequently proline and the -Yyy- position is frequently hydroxyproline. The tripeptide sequences with proline in the Xxx position and hydroxyproline in the Yyy position drive folding of the polypeptide chains into a unique triple-helical conformation of collagen. Other amino acids between the obligate glycine residues form hydrophobic or hydrophilic clusters on the surface of the molecules that direct the self-assembly of the proteins into stable fibrils. Because of the constraints conferred by the triple-helical conformation, the monomers of collagens are generally regarded as rigid rods. However, there are many indications that different regions of the collagen triple-helix vary in stability and undergo microfolding in the physiological range of temperatures (7–16). Evidence for the microfolding of the monomer included the effects of partially denaturing and then renaturing the protein (8, 9), experiments involving reversible inhibition of hydroxylation of proline and lysine residues during biosynthesis (10), comparisons of the helix-forming properties of synthetic peptides with repetitive -Gly-Xxx-Yyy- sequences (11–16), measurements of enthalpy changes by microcalorimetry (7), and the effects of temperature on the kinetics of fibril formation (17). One of the most direct indications of regions of varying stability in the triple helix came from mutations that convert different obligate glycine codons to codons for amino acids with bulkier side chains and cause the brittleness of bones and other tissues characteristic of the heritable disease known as osteogenesis imperfecta (3, 18–20). For example, one mutation that converted the glycine codon at position 631 of the α1(I) chain to a codon for serine had no measurable effect on the thermal stability of type I collagen, whereas a similar mutation that converted the glycine codon at position 598 to a codon for serine markedly lowered the melting temperature of the protein (20). Similar results were recently obtained with synthetic peptides containing collagen sequences in that a serine substitution for glycine at α1–913 destabilized the triple helix more than a serine for glycine substitution in the same peptide at α1–901 (16).

We recently developed a cDNA cassette system to synthesize recombinant versions of procollagen II with deletions in one of the four blocks of 234 amino acids that define the four repeating D periods of the collagen triple helix (21). Expression of the deleted D period cassettes in a mammalian system provided modified procollagen II that was secreted and was triple helical. Here we have compared the thermal stabilities of the recombinant procollagens missing specific D periods. The re-

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¶ Present address: Center for Gene Therapy, Allegheny University of the Health Sciences, 245 North 15 St., Rm. 10118 NCB, Mail Stop 421, Philadelphia, PA 19102.
sults provide direct evidence that some regions of the monomer are rich in sequences that stabilize the triple helix and thereby provide clamps for folding and unfolding of the molecule. The results also demonstrate that deletion of the sequences of the D1 period allows the protein to assume an unusual temperature-sensitive conformation at 35 °C so that procollagen N-proteinase cleaves an otherwise resistant Gly-Ala bond in the D2 period of the α1(II) chain.

MATERIALS AND METHODS
cDNA Constructs—Discrete regions of cDNAs for the pro-α1(II) chain of procollagen II were subcloned to generate seven cassettes that encoded (a) the N-propeptide and the N-telopeptide, (b) the D1 period, (c) the D2 period, (d) the D3 period, (e) the D4 period, (f) the 0.4 period at the C terminus of the triple helix, and (g) the C-telopeptide1 and the C-propeptide (21). The cassettes were amplified by the PCR using primers designed to introduce new restriction sites so that the cassettes could be assembled into a variety of different constructs (see Ref. 21). The DNA cassettes were cloned into the bacterial strain DH5α (Life Technologies, Inc.). The cassettes were excised with HindIII and EcoRI and then used to assemble a series of DNA constructs that were inserted into the HindIII and EcoRV sites of the mammalian expression vector pcDNA3 (Intronvert) containing a cytomegalovirus promoter and a gene encoding neomycin resistance. The structures of all the junctions in the constructs were verified by DNA sequencing.

Cell Transfection—HT-1080 cells (American Type Culture Collection, Rockville, MD) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (22). The cells were transfected with one of the functional DNA constructs by the calcium phosphate precipitation method using a commercial kit (Profection Mammalian Transfection System Kit; Promega). Briefly, cells were split 18 h prior to transfection, grown to a density of approximately 106 cells in a 10-cm cell culture dish, and provided with fresh culture medium 3 h prior to transfection. Each DNA construct used for transfection was linearized by cleavage with PvuII. Approximately 12 μg of DNA was precipitated with calcium phosphate and incubated with the cells for approximately 17 h. Fresh culture medium was then applied. After the cells reached confluency, they were split 1 to 10. After 24 h, medium was added that contained G418 (Life Technologies, Inc.) at an active concentration of 1 mg/ml. The medium was adjusted to pH 7.4. The cell lysate and proteins precipitated from the media were concentrated approximately 10-fold with filter cartridges with a 100-kDa molecular mass cut-off (PrepScale TFF; Millipore). The proteins were precipitated overnight by the addition of (NH4)2SO4 to a final concentration of 150 mg/ml, and the precipitate was collected by centrifugation at 15,000 × g for 1 h. Pellets from each 24-h collection were pooled and resuspended overnight in storage buffer (22) and then dialyzed against 200 volumes of DEAE-cellulose column III buffer (22) was used with minor modifications. Media harvested from each 24-h period was filtered through a 1.6-μm glass fiber filter (Millipore) and supplemented with stock solutions to provide the following final concentrations: 0.1 M Tris-HCl buffer, 0.4 M NaCl, 25 mM EDTA, 10 mM N-ethylmaleimide, 1 mM p-aminobenzamidine, and 0.04% Na3VO4. The proteins were precipitated overnight by the addition of (NH4)2SO4 to a final concentration of 175 mg/ml, and the precipitate was collected by centrifugation at 15,000 × g for 1 h. Pellets from each 24-h collection were resuspended overnight in storage buffer (22) and then dialyzed against 200 volumes of DEAE-cellulose column III buffer and then collected and dialyzed against 200 volumes of DEAE-cellulose column II buffer (22). The protein was dialyzed against 200 volumes of storage buffer and stored at ~80 °C. For further analysis by CD, the proteins were concentrated on a membrane filter (YM-100; Amicon) and the storage buffer was exchanged with storage buffer that did not contain EDTA.

Amino Acid Composition and N-terminal Amino Acid Sequence Analysis—The amino acid compositions and protein concentrations (mg/ml) of the purified recomagnanas were determined for each period construct on a Beckman 118 Model Amino Acid Analyzer. Purified human procollagen II was hydrolyzed for 24 h at 110 °C with 6 M HCl, and the amino acid composition was expressed as residues per mol chain. The amino acid sequence of the Gly-Ala bond at amino acid positions 393 and 394; RT-PCR, reverse transcriptase-polymerase chain reaction.

1 The abbreviations used are: C-telopeptide, C-terminal telopeptide; C-propeptide, C-terminal propeptide; N-telopeptide, N-terminal telopeptide; N-propeptide, N-terminal propeptide; CD, circular dichroism; FL, full-length procollagen II molecule; residues 1 to 1,014, amino acid positions 1-24; b, Flax-24 number of the first glycine in the major triple helix; pNae(1) chains, pro-α1(II) chains lacking the C-propeptide; pCa(1) chains, pro-α1(II) chains lacking the N-propeptide; pHαα, pro-α1(II) chains lacking the D1 period that are cleaved at the Gly-Ala bond at amino acid positions 394 and 395; RT-PCR, reverse transcriptase-polymerase chain reaction.

Purification of Recombinant Procollagens—The method of Fertala et al. (22) was used with minor modifications. Media harvested from each 24-h period was filtered through a 1.6-μm glass fiber filter (Millipore) and supplemented with stock solutions to provide the following final concentrations: 0.1 M Tris-HCl buffer, 0.4 M NaCl, 25 mM EDTA, 10 mM N-ethylmaleimide, 1 mM p-aminobenzamidine, and 0.04% Na3VO4. The proteins were precipitated overnight by the addition of (NH4)2SO4 to a final concentration of 175 mg/ml, and the precipitate was collected by centrifugation at 15,000 × g for 1 h. Pellets from each 24-h collection were pooled and resuspended overnight in storage buffer (22) and then dialyzed against 200 volumes of DEAE-cellulose column III buffer (22). The protein was dialyzed against 200 volumes of storage buffer and stored at ~80 °C. For further analysis by CD, the proteins were concentrated on a membrane filter (YM-100; Amicon) and the storage buffer was exchanged with storage buffer that did not contain EDTA.

Additional Assays for Expression of the ~D0.4 Period Construct—Because expression of the ~D0.4 period construct was not detectable by more rapid migration of the recombinant protein on electrophoretic gels, mRNA from cells expressing the construct was assayed by RT-PCR. Total mRNA was extracted from the cells with a selective resin (RNeasy; Qiagen), and the RNA was reverse transcribed with random primers (First Strand cDNA synthesis kit; Pharmac Biotech Inc.). The cDNA for pro-α1(II) chains amplified by PCR with a primer pair spanning the exon for Ser at amino acid position 898 (numbered from the first Gly of the triple helix) to the exon for Trp at amino acid position 1,136 in the C-propeptide. The PCR products were separated on an agarose gel.

To assay secretion of the protein, confluent clones expressing the FL and ~D0.4 constructs were incubated in 12-well microtiter plates (5 cm2) for 24 h (22). Proteins in the medium were precipitated with 5% polyethylene glycol. Samples were centrifuged at about 15,000 × g for 30 min at 4 °C and pellets were resuspended in 32 μl of storage buffer. Buffers used in 200 mM HEPES-NaOH buffer containing 0.1% deoxycholate, 0.1% Triton X-100, 10 mM EDTA, 0.5 unit of aprotinin/ml (Sigma), and 5% β-mercaptoethanol in phosphate buffer adjusted to pH 7.4. The cell lysates and proteins precipitated from the media were analyzed by Western blot analysis with guinea pig anti-human collagen II antibodies and secondary antibodies of anti-guinea pig IgG (H+L)/horse-
Recombinant Procollagens II

Cleavage of Recombinant Proteins with Procollagen N- and C-Proteinases—Purified 14C-labeled novel procollagens II were used as substrates for procollagen N-proteinase and procollagen C-proteinase purified from chick embryo tendons (23, 24). The assay conditions were 27 μl containing approximately 1 μg of procollagen and either 2.2 units of N-proteinase or 1.0 unit of C-proteinase in 7 mM CaCl2, 0.1 M NaCl, 0.015% Brij, and 0.02% NaN3 in 25 mM Tris/HCl buffer, pH 7.5. One unit of each of these enzymes is defined as the amount of enzyme needed to cleave 1 μg of substrate in 1 h at 35 °C using the buffer conditions just stated. Unless otherwise noted, digestions were generally performed at 35 °C for 3 or 4 h. Products of the proteinase cleavage were separated in SDS-polyacrylamide gels and analyzed using a phosphor storage plate.

Unfolding and Folding of the Recombinant Procollagens—CD was assayed in a spectropolarimeter (JASCO J-500A) using thermostatted quartz cells with a path length of 0.05 cm as described by Davis and Böhninger (13). The temperature of the sample was monitored by a thermistor and a digital thermometer (Omega Engineering, Inc.), and the temperature of the circulating water bath (Lauda RCS20D) was controlled by a temperature programmer (Lauda PM350). The CD spectrum of the sample was scanned from 180 to 260 nm. For melting experiments, the temperature of the sample was increased at a rate of 10 °C/h and the CD signal at 221 nm was monitored. The CD and temperature signals were recorded in the X-Y mode with an HP7090A measurement plotting system. The degree of conversion was calculated as described by Bruckner et al. (25). Because the protein yields were too low for assays by CD, the thermal stability of the procollagen lacking the D0.4 period was assayed by brief digestion with chymotrypsin and trypsin (26, 27). A clone secreting the protein was incubated with 14C-labeled amino acids, the medium proteins were protease digested, and the protease-resistant α1(II) chains were assayed by polyacrylamide gel electrophoresis in SDS followed by analysis with a phosphor storage plate analyzer (26, 27).

RESULTS

Recombinant Procollagens Lacking a Complete D Period—Cassettes of cDNA for the -D1, -D2, -D3, and -D4 periods were precipitated with 175 mg/ml ammonium sulfate, digested with chymotrypsin and trypsin to remove the propeptides and telopeptides, and were separated on 7.5% polyacrylamide gels in SDS. The gel was stained with Coomassie Blue. Recombinant collagen α chains with deletions of a complete D period migrate more rapidly than full-length α chains but differently from each other because of variations in post-translational modifications (22). The yield of secreted protein from clones expressing the -D0.4 construct were too low for purification of the recombinant protein. Therefore, the crude medium was precipitated with 175 mg/ml ammonium sulfate. The proteins were dialyzed and digested with 100 μg/ml trypsin and 250 μg/ml chymotrypsin at room temperature for 7 min before electrophoretic separation. The -D0.4 protein migrated similarly to FL chain. In addition, two bands of degradation products of α1(II) chains were seen (see Refs. 27–30).

Recombinant Procollagen Lacking the D0.4 Period—A sepa-
rate construct was prepared that lacked a cassette for the D0.4 period coding for the last 78 amino acids of the triple-helical domain of the α1(II) chain. Because deletion of 78 amino acids produced a minimal shift in migration of pro-α1(II) chains (Fig. 1), clones expressing the construct were identified by an RT-PCR. As expected, there was a difference of 234 base pairs in the RT-PCR products (top right panel in Fig. 3). Fewer positive clones were obtained than with constructs lacking a complete D period in that only 2% of stably transfected clones secreted detectable levels of recombinant protein (0.01 mg/ml), whereas 7–26% of clones obtained with the other constructs secreted high levels (0.1–0.2 mg/ml). The amount of intracellular pro-α1(II) and partially processed pro-α1(II) in clones expressing the D0.4 construct was about the same as in cells expressing the FL construct (top left panel in Fig. 3). However, the recombinant protein was not secreted as efficiently (middle panel in Fig. 3). As a result, the ratio of medium to intracellular recombinant protein after 24 h was 1.7 in the clones expressing the –D0.4 period construct whereas it was 23 in the clone expressing the FL construct. Because the yields were too low for assays by CD, the thermal stability of the procollagen lacking the D0.4 period was assayed by brief digestion with chymotrypsin and trypsin (26, 27). The assays indicated that the midpoint for unfolding of the recombinant procollagen II lacking the D0.4 period was about 3 °C lower than the full-length construct (lower panel in Fig. 3, and Table I).

Digestion of Recombinant Procollagens with C-Proteinase and N-Proteinase—Five of the purified 14C-labeled recombinant procollagens were tested as substrates for procollagen C-proteinase and N-proteinase (23, 24). The C-proteinase apparently cleaved all five of the recombinant proteins (Fig. 4). After digestion with the N-proteinase, fragments of the expected size were obtained with four of the procollagens, i.e. the FL protein and procollagens lacking either the D2, D3, or D4 periods (Fig. 4). However, procollagen lacking the D1 period generated a different pattern of fragments. Under a variety of conditions, the protein lacking the D1 period was digested more slowly (Figs. 4, 5 and 6). Also, two different large fragments were generated. One fragment co-migrated with the expected cleavage product of pCα1(II) chains. The fragment had the same N-terminal sequence as authentic pCα1(II) chains (24) and, therefore, arose from cleavage at the normal cleavage site of the N-proteinase. The second and more abundant fragment that was designated as pXα chains migrated more rapidly. Cleavage to pXα chains was temperature-dependent in that pXα chains were the major large fragment generated at 35 °C

| Procollagen | Unfolding by CD (°C) | Protease assay, Tm |
|-------------|---------------------|-------------------|
|             | Tm⁰.25 | Tm⁰.5 | Tm⁰.75 | Tm     |
| –D1         | 33.3   | 36.0  | 38.0  | 35.2   |
| –D2         | 37.9   | 39.3  | 40.4  |        |
| –D3         | 37.8   | 39.8  | 41.4  |        |
| –D4         | 35.3   | 39.6  | 40.6  |        |
| –D0.4       |        |       |       |        |
| FL          | 37.6   | 39.2  | 40.2  | 39.2   |

a Tm⁰.25, Tm⁰.5, and Tm⁰.75 correspond to the temperatures at which the degree of conversion is 0.25, 0.5, and 0.75, respectively. All assays were repeated at least two times. Significant differences from values for FL type II procollagen are in bold underlined type.

Recombinant Procollagens II

Table I

Thermal stability data obtained from CD analysis and protease digestion of recombinant type II procollagen

Fig. 2. CD melting curves of recombinant procollagens II. In each case the melting curves obtained for a procollagen lacking a complete D period is compared with that obtained for FL procollagen. CD absorbance data was collected at 221 nm. Samples were heated at a rate of 10 °C/h. Each melting curve contains 1000 data points. Symbols as in Fig. 1.
but pCα1(II) chains were the major products generated at 25 °C (Fig. 6). Specifically, the ratio of pCα1 to pXα chains was 3.4:1 at 25 °C, but decreased to 2.2:1 at 30 °C and to 0.2:1 at 35 °C. Cleavage to pXα chains was not explained by nonspecific cleavage of partially unfolded protein, since cleavage to pXα chains was not seen when the same protein was digested with a mixture of trypsin and chymotrypsin at temperatures ranging from 25 to 43 °C under the conditions of the experiment in Figure 3 (bottom panel). To define the structure of pXα chains, the band was excised from a polyacrylamide gel and the N-terminal sequence assayed. The sequence was Ala-Arg-Gly-Gln-Pro-Gly-Val-Met-Gly-Phe. Therefore, the cleavage by the N-proteinase was at the Gly-Ala bond between residues 394 and 395 of the α1(II) chain (31).

**DISCUSSION**

The results here extend previous indications that some sequences of the collagen molecule form a triple helix that is more thermally stable than other sequences in the same molecule (2, 4, 7–16). Since the procollagen II lacking the D1 period unfolded at a lower temperature than the FL molecule, the 234 residues in the D1 period must be rich in sequences that stabilize the triple helix and, therefore, serve as an N-terminal clamp for the helix. Since the procollagen II lacking the D2 period had the same melting profile as the full-length molecule,
the sequences between 235 and 468 must be relatively neutral in their effects. A similar conclusion was reached earlier on the basis of the observation that a spontaneous deletion of residues 157 to 447 did not significantly lower the melting temperature of a recombinant procollagen II (27). Since the procollagen II lacking the D3 period had a higher melting temperature than the FL molecule, the residues between 469 and 702 must contain sequences that are less helix stabilizing than most of the sequences in the molecule. With the procollagen II lacking the D4 period, the first 25% of unfolding occurred at a lower temperature than the full-length but the rest of the structure unfolded at the same temperature. The data suggest, therefore, that some of the sequences of residues 702 to 936 in the D4 period serve to stabilize the triple helix. The D4 period contains the sequences around the vertebrate collagenase site. With procollagen II lacking the 78 amino acids of the C-terminal D0.4 period (amino acids 937 to 1014), all of the secreted monomers unfolded 3 °C lower than the full-length protein. Therefore, the sequences of the D0.4 period that end in five triplets of -Gly-Pro-Hyp- (33) together with the C-terminal triplets of -Gly-Pro-Hyp- (33) together with the C-terminal triple helix. The presence of clamp-like sequences at both ends of the monomer of collagen II are consistent with previous observations on the kinetics of folding and unfolding of fragments of type III collagen (13).

Fewer clones expressing the −D0.4 construct were obtained and the yields of recombinant procollagen from the positive clones were far less than with the other constructs. Also, a smaller fraction of the −D0.4 procollagen was secreted into the medium. Therefore, the results indicated that the last 78 amino acids of the D0.4 period are required both for efficient folding and secretion of the protein, apparently because they are required for rapid nucleation of the triple helix. The results, therefore, complement the recent results of Bulleid et al. (34) who recently examined folding and assembly of procollagen in a semi-permeabilized cell system. They demonstrated that a single transmembrane domain can replace the roles of the C-propeptide and the C-telopeptide in chain association, but that a minimum of two hydroxyproline-containing Gly-X-Y triplets at the C terminus of the triple helix were required for nucleation.

To self-assemble into tightly packed and flexible fibrils, the collagen monomer must be synthesized not as a rigid rod but as a flexible structure that undergoes extensive microunfoldin in solution (1–4, 17). In mammals, the monomers completely unfold at about 41 °C and large regions demonstrate microunfoldin at 37 °C. At lower temperatures of 30–32 °C, the triple helix becomes more rigid, but the monomers of collagen II do not assemble into fibrils (35) and the monomers of collagen I form unusually thick and rigid fibrils (2, 17). Collagen monomers from poikilotherms consistently unfold at about 4 °C above body temperature (1). Therefore, there apparently has been selective pressure for about 500 million years of evolution (36) for synthesis of monomers that microunfold at the temperatures they self-assemble into fibrils. Probably, there is also selective pressure for clamp-like sequences at the ends of the monomers. The requirements both for regions of microunfoldin and for regions of more stable triple-helical conformation help to explain why some single amino acid substitutions in fibrillar collagens produce lethal phenotypes whereas the same amino acid substitutions at other sites in the same monomers produce milder phenotypes difficult to distinguish from osteo-

![Fig. 7. Schematic for the conformation of the N-propeptide in procollagen II. Upper panel, the full-length molecule at 35 °C. Middle panel, Procollagen lacking the D2 period at 35 °C. Bottom panel, Procollagen lacking the D1 period at 35 °C. Arrow indicates peptide bond cleaved by procollagen N-proteinase.](image-url)
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