Inhibition of the Self-assembly of Collagen I into Fibrils with Synthetic Peptides

DEMONSTRATION THAT ASSEMBLY IS DRIVEN BY SPECIFIC BINDING SITES ON THE MONOMERS

(Received for publication, December 30, 1997, and in revised form, March 20, 1998)

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A series of experiments were carried out to test the hypothesis that the self-assembly of collagen I monomers into fibrils depends on the interactions of specific binding sites in different regions of the monomer. Six synthetic peptides were prepared with sequences found either in the collagen triple helix or in the N- or C-telopeptides of collagen I. The four peptides with sequences found in the telopeptides were found to inhibit self-assembly of collagen I in a purified in vitro system. At concentrations of 2.5 mM, peptides with sequences in the C-telopeptides of the α1(I) and α2(I) chain inhibited assembly at about 95%. The addition of the peptide with the α2-telopeptide sequence was effective in inhibiting assembly if added during the lag phase and early propagation phase but not later in the assembly process. Experiments with biotinylated peptides indicated that both the N- and C-telopeptides bound to a region between amino acid 776 and 822 of the α(I) chain. A fragment of nine amino acids with sequences in the α2-telopeptide was effective in inhibiting fibril assembly. Mutating two aspartates in the 9-mer peptide to serine had no effect on inhibition of fibril assembly, but mutating two tyrosine residues and one phenylalanine residue abolished the inhibitory action. Molecular modeling of the binding sites demonstrated favorable hydrophobic and electrostatic interactions between the α2-telopeptide and residues 781-794 of the α(I) chain.

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Fibrillar collagens form the largest protein structures found in complex organisms (see Refs. 1 and 2). The most abundant collagen fibrils consist almost entirely of a single monomer of type I collagen. The structure of the monomer was established several decades ago, but the precise pattern of packing of the monomer into fibrils has not been defined and remains controversial (3-17).

Type I collagen is similar to other fibrillar collagen in that it is first synthesized as a soluble procollagen containing N-propeptides and C-propeptides (see Ref. 2). The propeptides are cleaved by specific N- and C-proteinases to generate the monomers that comprise collagen fibrils. The two α1(I) and one α2(I) chains of a monomer of type I collagen are primarily comprised of about 338 repeating tripeptide sequences of Gly-Xaa-Ybb in which Xaa is proline and Ybb is frequently hydroxyproline. The ends of the α1(I) and one α2(I) chains consist of short telopeptides of about 11–26 amino acids per chain. The distribution of hydrophobic and charged residues in the Xaa and Ybb positions in the triple-helical domain define 4.4 repeats or 4.4 D periods of about 234 amino acids each. In longitudinal sections, the monomers are arranged in fibrils in a head-to-head-to-tail orientation with a gap of about 0.6 D periods and, therefore, repeat of 5 D periods. The continuity of the fibrils is maintained by many of the monomers being staggered by 1, 2, 3, or 4 D periods relative to the nearest neighbor so as to generate gap and overlap regions. However, there are conflicting data from electron microscopy and x-ray analysis about the lateral packing of the monomers. One view is that the monomers are laterally packed in a tilted quasi-hexagonal lattice (4, 14). A related view is that the fibrils consist of “compressed” microfibrils that are comprised of monomers coiled into a rope-like pentameric structure (3, 6). Still another view is that the lateral packing of the collagen in many fibrils is either liquid-like or a biological equivalent of a liquid crystal (12, 13).

One experimental approach to defining the lateral packing of the monomers was to observe the initial assembly of monomers into fibrils. Early experiments (1, 11, 15) on the reassembly of fibrils from collagen extracted from tissues with acidic buffers suggested that the first structures formed were linear strands of monomers bound by 0.4 D period overlaps (4 D stagers). Other observations with extracted collagens suggested the initial stages involved assembly of structures similar to pentameric microfibrils (1, 15, 16). Subsequently, a system was developed for studying assembly of type I collagen fibrils de novo by enzymic cleavage of a purified soluble precursor of procollagen under physiological conditions (18-21). Because thick fibrils were generated in the system at 30–32 °C, it was possible to use dark-field light microscopy to follow the growth of the fibrils through intermediate stages (21). The first fibrils detected had a blunt end and a pointed end or tip. Initial growth of the fibrils was exclusively from the pointed or α tip. Later, β tips appeared on the blunt ends of the fibrils, and the fibrils grew from both directions. Scanning transmission electron microscopy indicated that both the α tips and β tips were near-paraboloidal in shape (22). Also, the monomers were oriented with their N termini directed toward the tips. Subsequent experiments in the same system with type II collagen suggested that the fibrils also grew from pointed tips. However, the monomers were oriented with a C termini directed toward the tips (23). Also, the fibrils contained central regions in which the monomers were packed symmetrically. Recent observations indicate there may be differences between collagen fibrils assembled in vitro and those assembled in vivo (24). In particular, collagen I fibrils assembled in vitro are exclusively bipolar, but fibrils from tissues are both bipolar and unipolar. Also, intermediates such as pNcollagen may participate in the initial steps of fibril assembly. All the data, however, are consistent

* These studies were supported in part by National Institutes of Health Grants AR39740 and RO43366. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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reaction mixture to final concentrations of 0.5–2.5 mM in 5 pCcollagen I, and 15 units/ml procollagen C-proteinase purified from m
After incubation for 0.5 to 24 h at 37 °C, the sample was centrifuged
was assayed in a gel filtration column. Fibril assembly was assayed under conditions employed previously (18–21, 23). In brief, the
was synthesized and purified by a commercial concern (American Peptide Com-
fibrils depends on the interactions of specific binding sites in
were accounted for x-ray diffraction data that indicated that some fraction of monomers in fibrils were laterally packed in a tilted quasihexagonal lattice (4, 14). In contrast to the first two models, a third model (27) was developed in which monomers were assembled by a process involving only aggregated limited diffusion. The third model, therefore, assumed that the assembly of monomers into fibrils was similar to processes such as electrochemical depositions or perhaps formation of snowflakes and that the process did not require the presence of specific binding sites on the monomers.
Here, we have developed a series of experiments on the assumption that the assembly of collagen I monomers into fibrils depends on the interactions of specific binding sites in different regions of the monomers. To test this assumption, we examined the effects of synthetic peptides on the assembly of fibrils de novo.
MATERIALS AND METHODS
Design and Synthesis of Peptides—The synthetic peptides were synthesized and purified by a commercial concern (American Peptide Company, Inc.). Homogeneity and stability of the peptides in experimental conditions were assayed by reverse-phase HPLC. All the peptides were freely soluble in the fibril formation buffer. The pH of peptide solutions was monitored with a solid state micro-pH electrode (Beckman).
Assembly of Fibril Assembly—Assembly of collagen I into fibrils de novo was assayed under conditions employed previously (18–21, 25). In brief, 14C-labeled type I procollagen was recovered from the medium of cultured human skin fibroblasts and was purified with two chromatographic steps to homogeneity. The type I procollagen was processed to pCollagen I by cleavage with procollagen N-proteinase purified from organ cultures of chick embryo tendons. The pCollagen was then isolated on a gel filtration column. Fibril assembly was assayed in a 20-μl reaction volume in a 250-μl plastic centrifuge tube sealed with a plunger and containing a physiological bicarbonate buffer, 30 μg/ml pCollagen I, and 15 units/ml procollagen C-proteinase purified from chick embryo tendons. Potentially inhibitory peptides were added to the reaction mixture to final concentrations of 0.5–2.5 μM in 5 μl of buffer. After incubation for 0.5 to 24 h at 37 °C, the sample was centrifuged 13,000 × g for 10 min. The pellet and supernatant fractions were separated by electrophoresis on a 7.5% polyacrylamide gel in SDS, and the gel was assayed either with a phosphor storage imager (STORM, Molecular Dynamics) or by staining with colloidal Coomassie Blue (Brilliant Blue; Sigma) and analysis with a densitometer (Personal Densitometer, Molecular Dynamics). Alternatively, the reaction was carried out in a sealed chamber on a microscope slide and followed by dark-field light microscopy (Zeiss model 009).
Localization of Binding Sites on Collagen—Type I collagen extracted with 0.5 M acetic acid from mouse skin was digested with pepsin, and the α chains were separated by gel electrophoresis in SDS. To generate CNBr peptides, gel slices containing α1(I) and α2(I) chains were placed into tubes, and α chains were digested with 10 mg/ml or 200 mg/ml CNBr in 70% formic acid at room temperature overnight. The gel slices were equilibrated with 0.05 mm Tris-HCl buffer (pH 6.8) over 3 h. Gel pieces containing CNBr peptides derived from individual collagen α chains were transferred into the wells of the second gel prepared with a 6% polyacrylamide stacking gel and 12% polyacrylamide separating gel with 0.5 μt urea. After electrophoresis, the gel was electroblotted overnight onto a nitrocellulose filter at 4 °C onto a nitrocellulose filter (Millipore). To examine the binding of telopeptides to collagen fragments, the method described by Fujisawa et al. (28) was used. The filters were blocked with 1% bovine serum albumin (Sigma) and then incubated overnight with 20 ml of 5 μg/ml peptide that was substituted at the N terminal with biotin (synthesized for us by American Peptide Company, Inc.). The filter was washed three times with Tris-buffered saline containing 0.02% Tween 20 and incubated 30 min with a 1:30,000 dilution of horseradish peroxi-
dase conjugated with avidin (Sigma). The bands were detected by chemiluminescence (ECL; Amersham Pharmacia Biotech) after exposure to an x-ray film for 3 to 10 min.
To generate vertebrate collagenase fragments, 3 μg of type I pCollagen from cultured human skin fibroblasts was cleaved with 10 μg/ml of vertebrate collagenase from cultured rat skin fibroblasts (generous gift from John J. Jeffrey, Department of Medicine, Albany Medical College) for 3 h at 25 °C in a volume of 40 μl of 50 mM Tris-HCl, 10 mM CaCl2, and 100 mM NaCl, pH 7.4. To remove the C-propeptide, 2 μl of a mixture of trypsin (1 mg/ml) and chymotrypsin (2.5 mg/ml) in the same buffer was added, and the sample was incubated at 20 °C for 2 min. The reaction was stopped with 0.5 mg/ml soybean trypsin inhibitor (Sigma). The sample was then sepa-
rated by electrophoresis on a 10% polyacrylamide gel in SDS and pro-
cessed with the same protocol as the CNBr fragments.
Competition Assay—To define the telopeptide binding site with a competitive assay, 96-well titration plates (Immulon 3, Dynatech Laboratories, Inc.) were used. Seventy microliters of the solution containing 3 μg of pepsin-treated monomeric collagen (Vitrogen 100, Collagen Biomaterials) in 0.1 x HCl was added to each well, and the plates were dried at room temperature. As a control, wells covered with bovine serum albumin were used. The plates were rinsed with sterile phosphate-buffered saline and nonspecific binding sites were blocked with 1% bovine serum albumin. For the competition assay, pre-mixed samples were prepared that contained 50 μt biotinylated F2 or F3 peptide with 10–500 μt peptide α1-776/796. As a negative control, peptide α2-218/233 (F1) was used at concentrations from 10 to 500 μM. The peptide mixtures were added into wells containing immobilized colla-
gen, and the samples were incubated at 25 °C for 12 h. The plates were washed with PBS containing 0.05% Tween. To detect biotinylated peptides bound to collagen surface, streptavidin conjugated to alkaline phosphatase (Bio-Rad) was added at 1:20,000 dilution. After 2 h of incubation, the plates were washed with PBS containing 0.05% Tween, and the developing reagent 3 mM p-nitrophenyl phosphate in 0.05 M Na2CO3, 0.05 mM MgCl2, (pH 9.5) was added. After 2 h of incubation, the reaction was measured with microtiter plate reader (Dynatech Labora-
tories) using a 405-nm filter.
Computer Modeling of Binding Sites—Molecular modeling was performed on a Silicon Graphic (Indigo 2) computer system using the SYBYL software package, Version 6.3 (Tripos, Inc.). The model of the collagen I triple helix fragment including sequence from α1-766/801 and α2-766/801 was carried out as described by Chen et al. (29). The model of F7 peptide was created using SYBYL/Biopolymer module. All the models were energy-minimized using a conjugate gradient method and subjected to repeating cycles of molecular dynamics using Kollman united-atom force field and united atoms (30). To analyze interaction of F7 peptide with the collagen I binding region, intermolecular energy of interaction was analyzed to identify possible binding conformations. Surface calculations, lipophilicity potential, and electrostatic potential of the molecule were analyzed using SYBYL/Molcad module.
RESULTS
Assays of Inhibition of Fibril Formation with Synthetic Peptides—Six synthetic peptides (Fig. 1) were prepared on the basis of two general considerations. (a) Extensive previous work (see Ref. 1) had demonstrated that the telopeptides were required to be present on the monomers to generate tightly packed fibrils, and (b) the self-assembling of collagen monomers is driven by electrostatic interactions (see Ref. 19), and therefore, any specific binding sites in the triple helix are likely to be found in hydrophobic sequences. As indicated in Fig. 1, one peptide (F1) contained relatively hydrophobic sequences found near the end of the D1 period of the α2(I) chain, a second peptide (F5) had hydrophobic sequences found near the middle of the D4 period of the α2(I)
1 The abbreviation used is: HPLC, high performance liquid chromatography.
chain. F6 contained sequences that spanned the vertebrate collagenase cleavage site of Gly-Ile in the D4 period of the α1(I) chain. The remaining three peptides had the sequences of the α1N-telopeptide, α1C-telopeptide, and α2C-telopeptide. Assays by reverse-phase HPLC demonstrated that none of the peptides degraded under experimental conditions used here (data not shown). The peptides were added in concentrations of 2.5 mM to the system for assaying fibril formation by the cleavage of pCollagen with C-proteinase (18–21, 23). As indicated in Fig. 2 and Table I, the peptides F1 and F6 caused little if any inhibition of fibril assembly. Peptides F2, F3, and F4 almost completely inhibited fibril assembly, whereas F5 inhibited assembly by about 50% under the conditions of the experiment. Inhibition of fibril assembly with the peptide F3 was directly demonstrated by following the reaction in a sealed chamber with dark-field light microscopy. No fibrils appeared when F3 was present in a concentration of 2.5 mM (Fig. 3, right panel). The specificity of the effects was demonstrated by preparing a peptide in which the same amino acids found in F3 were assembled in a random sequence. As indicated in Fig. 2 (bottom panel), the peptide with the scrambled sequence (SC/F3) had no effect on fibril assembly. Assays in which the concentration of the peptide F3 were varied indicated that fibril assembly was inhibited about 45% with a concentration of about 1.5 mM and almost 100% with 2.5 mM (Table I).

The α2C-telopeptide (F3) Inhibits Fibril Assembly if Added to the Lag Phase and Early Propagation Phase—To define the kinetics for inhibition of fibril assembly by the telopeptides, the peptide F3 was added in a concentration of 2.5 mM during the lag phase, early propagation phase, mid-propagation phase, or late propagation phase of fibril assembly (Fig. 4). The peptide inhibited fibril formation if added during the lag period. It partially inhibited if added during the early propagation phase, and it had little of any effect if added during the mid-propagation phase of fibril assembly.

Location of the Telopeptide Binding Site in the Triple Helix of Collagen I—To test the specificity of the binding of the peptides to collagen, a series of hybridizations were carried out with filter blots of proteins and a biotinylated derivative of the peptide F3. As indicated in Fig. 5, the biotinylated peptide F3 did not bind to any component of bovine serum. However, it bound to α1(I) chains, β chains that included α1(I) chains, pCα1(II) chains, and pCα1(III) chains. The same peptide bound to CNBr peptides (Fig. 6). As indicated in Fig. 6B, the biotinylated derivatives bound to the α1(I) chain but not the α2(I) chain. As also indicated in Fig. 6B, the biotinylated telopeptides bound to CB7 of the α1(I) chain that contains amino acid residues 552–822. There was no apparent binding to any of the other cyanogen bromide fragments.

To further define the binding site in the triple helix, vertebrate collagenase A and B fragments of type I collagen were prepared and hybridized with the biotinylated telopeptides. As indicated in Fig. 7 (middle and right panels), the peptides bound specifically to the B fragment of the α1(I) chain. Since vertebrate collagenase cleaves the two α chains of type I collagen between residues 775 and 776 (1), the binding of the peptides to both CB7 and the B fragments of the α1(I) chain indicated that the binding site is between amino acid 776 and 822 of the α1(I) chain.

Defining the Critical Residues in the α2C-telopeptide (F3)—Further experiments were primarily concentrated on the F3 peptide with the sequence of the C-telopeptide, because it was relatively short and somewhat repetitive in sequence (Fig. 1). To define the critical sequences within the peptide F3, several derivatives were prepared. Two peptides that were 9-mers overlapping the central region of the sequence (F7 and F8) were equally effective as the intact F3 (Figs. 1 and 8 and Table I).
Smaller effects on inhibition of fibril assembly were seen with two other fragments (F9 and F10) (Figs. 1 and 7 and Table I). Of special interest was that mutating the two aspartates in a 9-mer peptide (F7) to serine residues (F7/A) had no effect on inhibition of fibril assembly. However, mutating two tyrosines and one phenylalanine in the same sequence to serine residues (F7/B) abolished inhibitory effects (Figs. 1 and 8 and Table I).

Defining the Critical Sequences in the α1(I) Chain—To map the binding site in the triple helix still further, a peptide was prepared with the sequence of the amino acids in positions 776 to 796 of the α1(I). The sequence 776 to 796 was selected primarily because it was the most hydrophobic sequence in the region between 776 and 822 that was defined by the experiments with the CNBr peptides and the collagenase fragments (Fig. 9). The peptide α1–776/796 was then used in a competition binding assay in which collagen was bound to the wells of

| Peptide                        | Region of sequence | Code | Concentration | % Inhibition |
|--------------------------------|--------------------|------|---------------|--------------|
| Triple helix                   |                    |      |               |              |
| α2–217/233                     |                    | F1   | 2.5           | 5            |
| α2–778 to 801                  |                    | F5   | 2.5           | 50           |
| α1–761 to 785                  |                    | F6   | 2.5           | 0            |
| Telopeptides                   |                    |      |               |              |
| α1N-telopeptide                |                    | F4   | 2.5           | 90           |
| α1C-telopeptide                |                    | F2   | 2.5           | 95           |
| α2C-telopeptide                |                    | F3   | 2.0           | 70           |
| α2C-telopeptide                |                    | F3   | 1.5           | 45           |
| α2C-telopeptide                |                    | F3   | 0.5           | 25           |
| Derivations of α2C-telopeptide |                    |      |               |              |
| GGYDFGYD                      |                    | F7   | 2.5           | 90           |
| GYDGDFYRA                     |                    | F8   | 2.5           | 90           |
| GYDFGYDGDF                    |                    | F9   | 2.5           | 70           |
| DFRYRA                        |                    | F10  | 2.5           | 70           |
| GGGYSFGYS                     |                    | F7/A | 2.5           | 90           |
| GGGSDSGSD                     |                    | F7/B | 2.5           | 0            |

FIG. 3. Darkfield analysis of collagen I fibrils. pCcollagen was incubated with C-proteinase at 37 °C for 24 h in a sealed chamber in the presence (+) or absence (−) of 2.5 mM F3 peptide. Fibrils were photographed using a microscope with dark-field attachment. Magnification, 300×.

FIG. 4. Effects of adding the peptide F3 at varying time points during fibril assembly. Assays as in Fig. 2. Symbols: □, fibril assembly under control conditions; ▲, arrows, times when 2.5 mM F3 was added to parallel samples and then the incubation continued for 24 h; ●, values observed at 24 h.

FIG. 5. Binding of the biotinylated peptide F3 to filter blots of proteins. The proteins were separated by polyacrylamide gel electrophoresis in SDS and electroblotted onto filters. The filters were then hybridized with the biotinylated α2C-telopeptide followed by assay of the washed filter with streptavidin alkaline phosphatase. Left panel, filter stained with Coomassie Blue. Right panel, filters hybridized with biotinylated F9. Lane 1, pepsinated type I collagen from bovine skin. Lane 2, bovine serum proteins. Lane 3, pCcollagen II. Lane 4, pCcollagen I.

FIG. 6. Binding of biotinylated telopeptides to the α1(I) chain and the CB7 fragment chain. Conditions as in Fig. 5. Panel A, Coomassie Blue-stained filter of α1(I) and α2(I) chains and CNBr peptides. Panel B, filters probed with biotinylated telopeptides and streptavidin conjugated to peroxidase. Symbols: CNBr +, α1(I) or α2(I) chains digested with cyanogen bromide; F2, F3, F4, and NA2, separate filters probed with biotinylated C-telopeptide of the α1(I) chain, C-telopeptide of α2(I) chain, N-telopeptide of α1(I) chain, and N-telopeptide of α2(I) chain.
specific binding sites on collagen I monomers

FIG. 7. Binding of biotinylated telopeptides to collagenase A and B fragments of type I collagen. Left panel: Coomassie-stained polyacrylamide gel of α1(I) and α2(I) chains and vertebrate collagenase fragments. Middle and right panels, filters probed with biotinylated telopeptide and streptavidin conjugated to horseradish peroxidase. Symbols: VC −/+, samples with or without prior digestion with vertebrate collagenase; T, CH, bands of trypsin and chymotrypsin used to convert the procollagen into collagen. As indicated, there is some reactivity of the biotinylated telopeptides with large amounts of trypsin and chymotrypsin. Other symbols as in Fig. 6.

FIG. 8. Assay of inhibition of fibril formation with peptides that are fragments and modified version of the α-telopeptide (F3). See Fig. 1 and Table I for summary. Symbols: P, pellet fraction; S, supernatant fraction; C, control sample.

The results here resolve a critical question about the self-assembly of type I collagen into fibrils. If the assembly does not depend on specific interactions of binding sites in the monomers, as recently suggested by Parkinson et al. (27), all the peptides tested here should either have had no effect on fibril assembly or inhibited the process to about the same degree. Instead, the results demonstrated that several peptides specifically inhibited the process. Consistent with previous observations (see Ref. 1), peptides with sequences found in the telopeptides were the most effective. The telopeptide that was most extensively studied, the C-telopeptide of the α2(I) chain, completely prevented fibril assembly if added at or before the first one-third of the lag phase but had much less effect thereafter. Therefore, the binding of the α2C-telopeptide, probably in concert with α1C-telopeptide, is critical for early steps in the assembly process such as formation of a structural nucleus that is essential for further growth of the fibrils. The binding of the C-telopeptides to the region that encompasses residues 776 to 796 places the monomers in quarter D-period stagger (Fig. 9). Therefore, the binding could initiate assembly of a Smith-type pentameter microfibril (see Refs. 1 and 25). In contrast, the binding of the N-telopeptides to about the same region of the α1(I) chain (defined here as residues 776 to 822) places the monomers in quarter D-period stagger (Fig. 9). According to the model, the binding could initiate assembly of a Smith-type pentameter microfibril (see Refs. 1 and 25). In contrast, the binding of the N-telopeptides to about the same region of the α1(I) chain (defined here as residues 776 to 822) places the monomers in quarter D-period stagger (Fig. 9).

FIG. 9. The Figure shows the alignment of the N-telopeptides to the region that encompasses residues 776 to 796 places the monomers in quarter D-period stagger. The binding could initiate assembly of a Smith-type pentameter microfibril (see Refs. 1 and 25). In contrast, the binding of the N-telopeptides to about the same region of the α1(I) chain (defined here as residues 776 to 822) places the monomers in quarter D-period stagger (Fig. 9). Therefore, the binding could initiate assembly of a Smith-type pentameter microfibril (see Refs. 1 and 25). In contrast, the binding of the N-telopeptides to about the same region of the α1(I) chain (defined here as residues 776 to 822) places the monomers in quarter D-period stagger (Fig. 9).

Molecular Modeling of the Two Binding Sites—To model the binding of one of the telopeptides to the triple helix, the SYBYL program was first used to model the conformation of one of the nine-amino acid fragment (F7) of the α2C-telopeptide that inhibited fibril assembly (Figs. 1 and 8 and Table I). Because the aromatic Tyr and Phe residues tended to form a hydrophobic stack on one side of the polypeptide chain, a single conformation was favored (Fig. 11, A and B).

Binding of F7 to the region between residues 781 to 794 of α1(I) chain was analyzed. The possible binding conformation was interactively identified using the DOCK command. The refined model demonstrated a favorable interaction of hydrophobic groups (Fig. 11A) and electrostatic groups (Fig. 11B).

DISCUSSION

The results here resolve a critical question about the self-assembly of type I collagen into fibrils. If the assembly does not depend on specific interactions of binding sites in the monomers, as recently suggested by Parkinson et al. (27), all the peptides tested here should either have had no effect on fibril assembly or inhibited the process to about the same degree. Instead, the results demonstrated that several peptides specifically inhibited the process. Consistent with previous observations (see Ref. 1), peptides with sequences found in the telopeptides were the most effective. The telopeptide that was most extensively studied, the C-telopeptide of the α2(I) chain, completely prevented fibril assembly if added at or before the first one-third of the lag phase but had much less effect thereafter. Therefore, the binding of the α2C-telopeptide, probably in concert with α1C-telopeptide, is critical for early steps in the assembly process such as formation of a structural nucleus that is essential for further growth of the fibrils. The binding of the C-telopeptides to the region that encompasses residues 776 to 796 places the monomers in quarter D-period stagger (Fig. 9). Therefore, the binding could initiate assembly of a Smith-type pentameter microfibril (see Refs. 1 and 25). In contrast, the binding of the N-telopeptides to about the same region of the α1(I) chain (defined here as residues 776 to 822) places the D-periods out of register by one-third or more of a D-period of 294 residues. Therefore, the binding of the N-telopeptides cannot generate the 0D, 1D, 2D, 3D, and 4D stagger that are found among many nearest neighbors in fibrils assembled in vivo. Also, the binding of the N-telopeptides does not accurately align the monomers for formation of the major covalent cross-link that forms between the Lys residue at position 9 of the N-telopeptide and the Lys residue at 930 of the α1(I) chain (1, 36). Accordingly, there are several possible explanations for the observed binding of the N-telopeptides. One is that the binding occurs only with linear N-telopeptides such as those used here but not with N-telopeptides in the unusual hairpin conformation that is present in the native molecule (31–35) and that is essential both for assembly into well ordered fibrils and correct cross-linking (36). A second explanation is that binding of the N-telopeptides generates aberrant structural nuclei that cannot grow into fibrils and that resembles the “overshoot” struc-
tures seen in the assembly of tobacco mosaic virus (37). Preliminary assays with an optical biosensor indicated that the dissociation constant for the binding of the C-telopeptide is about $5 \times 10^{-6}$ M, and the dissociation constants of the two N-telopeptides are about an order of magnitude greater. Therefore, aberrant structures assembled by binding through the N-telopeptides may have a short half-life and may rapidly dissociate into monomers that initiate fibril assembly through binding of C-telopeptides. A third possibility is that binding through the N-telopeptides does not play an important role in fibril assembly until a core of a microfibril is formed, and it is only important for lateral growth of the fibril. The last suggestion is consistent with one of the proposed helical models for growth of microfibrils from paraboloidal tips (25). The model required one specific binding step governed by one rate constant ($k_1$) for assembly of monomers in a 1D-stagger to form a Smith-type microfibrillar core and to regulate longitudinal growth of the fibril. It required a second binding step governed by a smaller rate constant ($k_2$) to initiate growth of a new layer of helical sheets of monomers on the microfibrillar core and thereby to regulate lateral growth of the core.

The results here demonstrated that the binding of the C-telopeptide of the $\alpha_2(I)$ chain to residues 776 to 796 of the $\alpha_1(I)$ chain was directed primarily by hydrophobic interactions, since mutating two tyrosine residues and one phenylalanine residue in a nine-amino acid fragment abolished all effects on fibril formation, whereas mutating two aspartate residues had no effect. The modeling experiments indicated that there were conformations of the peptide and the triple helix that allowed good hydrophobic and electrostatic interactions between the nine-amino acid fragment C-telopeptide and the region between 776 and 796. The region contains the C-terminal half of the vertebrate collagenase cleavage site that previously has been designated as a relatively flexible region of the collagen triple helix (38). Also, Bhatnagar et al. (39) recently examined a synthetic peptide with amino acid residues 776 to 780 from the region and found that it had a high potential to form a stable $\beta$-bend with the central GIAG sequence that begins in residues 775. Moreover, they demonstrated (38) that the peptide inhibited the binding of fibroblasts to collagen at a concentration of $7.2 \times 10^{-6}$ M. Therefore, the sequence of amino acids in the region may take part in a large number of different binding interactions.

Finally, it is apparent that given the specificity of the binding interactions, the sites defined here provide interesting targets for peptides, peptidomimetics, or related compounds that may inhibit the assembly of collagen fibers in pathologic fibrotic conditions. The ability to model binding sites in the triple helix and the conformation of short fragments of the telopeptides provides a rational route for developing inhibitors.

\[ \text{FIG. 9. Schematic of the binding site in the triple helical domain of the } \alpha_1(I) \text{ chain as defined by the experiments with CNBr and vertebrate collagenase fragments (Figs. 6 and 7). Bottom of figure indicates that the binding of the C-telopeptides to the region is consistent with one quarter D stagger of monomers and fibrils. The underline sequence of } \alpha_1–776 \text{ to 796 was used as competitive peptide to further define the binding site (see Fig. 10).} \]

\[ \text{FIG. 10. Competition by the peptide } \alpha-776/796 \text{ for the binding of the C-telopeptides to type I collagen. Type I collagen was bound to microtiter plates and then incubated with a mixture of a biotinylated telopeptide and varying concentrations of the competitor peptide } \alpha_1–776/796. \text{ The washed filters were then assayed by reaction with streptavidin-alkaline phosphatase. Left panel, competition for binding of the biotinylated C-propeptide of the } \alpha_1(I) \text{ chain (peptide F2). Right panel, competition for the binding of the C-telopeptide from the } \alpha_2(I) \text{ chain (peptide F3).} \]

\[ \text{2 A. Fertala and D. J. Prockop, manuscript in preparation.} \]
competitive assays on microtiter plates provide a means of high throughput screening for large libraries of potential inhibitors.

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*J. Biol. Chem.* 1998, 273:15598-15604.
doi: 10.1074/jbc.273.25.15598

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