A Type I Collagen with Substitution of a Cysteine for Glycine-748 in the α1(I) Chain Copolymerizes with Normal Type I Collagen and Can Generate Fractallike Structures†

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ABSTRACT: Type I procollagen was purified from cultured fibroblasts of a proband with a lethal variant of osteogenesis imperfecta. The protein was a mixture of normal procollagen and mutated procollagens containing a substitution of cysteine for glycine in either one proα1(I) chain or both proα1(I) chains, some or all of which were disulfide-linked through the cysteine at position α1–748. The procollagen was then examined in a system for generating collagen fibrils de novo by cleavage of the pCcollagen to collagen with procollagen *C*-proteinase [Kadler et al. (1987) *J. Biol. Chem. 262*, 15696–15701]. The mutated collagens and normal collagens were found to form copolymers under a variety of experimental conditions. With two preparations of the protein that had a high content of α1(I) chains disulfide-linked through the cysteine α1–748, all the large structures formed had a distinctive, highly branched morphology that met one of the formal criteria for a fractal. Preparations with a lower content of disulfide-linked α1(I) chains formed fibrils that were 4 times the diameter of control fibrils. The formation of copolymers was also demonstrated by the observation that the presence of mutated collagens decreased the rate of incorporation of normal collagen into fibrils. In addition, the solution-phase concentration at equilibrium of mixtures of mutated and normal collagens was 5–10-fold greater than that of normal collagen. Therefore, the mutated and normal collagens synthesized by the proband's fibroblasts were similar enough in structure to copolymerize into the same fibrils, and because of copolymerization, there was a distortion of normal fibril morphology, a delay in fibril formation, and a decrease in the net amount of collagen incorporated into fibrils. The results were consistent with a recently proposed model for growth of collagen fibrils from pointed tips [Kadler et al. (1990) *Biochem. J. 268*, 339–343].

Previously we described a novel system of generating collagen fibrils de novo (Miyahara et al., 1982, 1984; Kadler et al., 1987, 1988). Key features of the system are that the monomer for fibril assembly is generated by enzymic cleavage of a soluble precursor of collagen, the fibrils are formed in a physiological buffer and over a physiological range of temperatures, and the fibrils formed at 37 °C are as round, thin, and apparently as flexible as those found in vivo (Kadler et al., 1990a). The system made it possible to demonstrate that the initial fibrils formed had blunt and pointed ends and that the fibrils grew from the highly tapered and tightly packed pointed ends in which the N-termini[1] of the molecules were oriented toward the tip (Kadler et al., 1990b). After a time, needlelike projections appeared at the blunt ends and then developed into growing tips with the N-termini of the molecules again oriented toward the pointed tips. The fibrils were, therefore, asymmetric and had a maximal diameter closer to one end of the fibril. The asymmetric growth of collagen fibrils was consistent with recent observations by Birk et al. (1989), who defined the ends of individual type I collagen fibrils by analyzing electron micrographs of serial sections of leg tendons from chick embryos.

Mutations that change the primary structure of type I procollagen cause osteogenesis imperfecta, a heritable disorder characterized by brittleness of bone [see Prockop et al. (1989), Byers (1990), and Kuivaniemi et al. (1991)]. Recently, we found that a proband with a lethal form of the disease was heterogeneous for a point mutation in the proα1(I) gene for type I procollagen (COL1A1) that changed the codon for glycine α1–748[2] to a codon for cysteine (Vogel et al., 1987). The type I procollagen secreted by the proband's fibroblasts in culture was a mixture of normal procollagen, procollagen containing one mutated proα1 chain, and procollagen containing two mutated proα1 chains, some or all of which were linked by disulfide bonds through the cysteine at α1–748. Examination of the procollagen by rotary-shadowing electron microscopy demonstrated that many of the molecules had a

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[1] Abbreviations: N- and C-, NH_2- and COOH-terminal, respectively; pCcollagen, intermediate in the conversion of procollagen to collagen containing the C-terminal propeptides but not the N-terminal propeptides; pCα chains, polypeptides of pCcollagen; α chains, polypeptides of collagen; SDS, sodium dodecyl sulfate.

[2] Amino acid positions are numbered with the standard convention in which the first glycine of the triple-helical domain of an α chain is number 1. The numbers for α1(I) chains can be converted to positions in the human proα1(I) chains by adding 156.

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flexible kink at the site of the cysteine substitution (Vogel et al., 1988). Here we used the system for assembling of collagen fibrils de novo to test the hypothesis that the mutated collagens generated by enzymic processing of the cysteine-substituted procollagens may form copolymers with normal type I collagen.

Materials and Methods

Suppliers of Materials. Mixtures of uniformly ^{14}C-labeled L-amino acids were purchased from ICN Radiochemicals; Dulbecco's modified Eagle's medium and fetal bovine serum were from GIBCO; DEAE-cellulose and preswollen microgranular anion exchanger DE-52 were from Whatman Biosystems Ltd.; Sephacryl S-300 Superfine gel filtration medium was from Pharmacia–LKB Biotechnology; other reagents were from Sigma Chemicals, ICN Biochemicals, and Bethesda Research Laboratories.

Preparation of Procollagens. Type I procollagens were purified from the medium of cultured skin fibroblasts from normal human skin and from the skin of a proband with a lethal form of osteogenesis imperfecta (Vogel et al., 1987, 1988). The proband was a male fetus aborted at 27 weeks of pregnancy after a routine ultrasound examination disclosed short and seriously malformed limbs (Vogel et al., 1987). Normal control fibroblasts were from a 10-year-old donor (GM 3349; Human Genetic Mutant Cell Repository, Camden, NJ) and a 31-year-old donor (JIMM-92, Jefferson Institute of Molecular Medicine, Philadelphia, PA). The passage numbers of the cells varied from 6 to 13.

Type I procollagen was purified from the cells in culture as previously described (Kadler et al., 1987, 1988). In brief, cells were grown to confluency at 37 °C in Dulbecco's modified Eagle's medium containing 1 μCi·mL^{-1} of a mixture of L-^{14}C-amino acids, 25 μg·mL^{-1} L-ascorbic acid, and no fetal bovine serum. Proteins were precipitated from the medium by ammonium sulfate, and the type I procollagen was purified by chromatography on two successive columns of DEAE-cellulose (Peltonen et al., 1980; Fiedler-Nagy et al., 1981). Purified procollagen was pressure-concentrated on a membrane (YM100, Amicon) and stored at −15 °C in 0.1 M Tris-HCl buffer containing 0.4 M NaCl and 0.02% NaN_3, pH 7.4 at 20 °C. Procollagen concentration was determined by a colorimetric assay for hydroxyproline using either the procedure of Kivirikko et al. (1967) or the procedure of Woessner (1961), and assuming 10.1% hydroxyproline by weight for type I procollagen (Fiedler-Nagy et al., 1981). The specific activities were 1500–2600 cpm·μg^{-1} for the control procollagen and 1600–2500 cpm·μg^{-1} for the proband's procollagen.

Preparation of pCcollagen and Procollagen C-Proteinase. Type I pCcollagen was prepared by digesting type I procollagen with procollagen *N*-proteinase. The *N*-proteinase was partially purified from chick embryos as described by Hojima et al. (1989). Digestion was stopped by adding one-tenth volume of 0.25 M EDTA and 0.1% NaN_3 in 1 M Tris-HCl buffer (pH 7.4 at 20 °C). To purify the pCcollagen, the protein was chromatographed on a column of Sephacryl S-300 (Kadler et al., 1987) and then on a column of DEAE-cellulose. Before chromatography on the DEAE-cellulose column, the pooled fractions from the S-300 column were dialyzed twice against 2 M urea and 0.01% NaN_3 in 0.075 M Tris-HCl buffer (pH 7.8 at 20 °C). The pCcollagen from the control and proband appeared in the flow-through fractions of the column. Type I procollagen that remained after incubation with *N*-proteinase was bound by the column. The pCcollagen was dialyzed against 0.4 M NaCl and 0.04% NaN_3 in 0.1 M Tris-HCl buffer (pH 7.4 at 20 °C) and concentrated by pressure ultrafiltration on a membrane (YM100, Amicon).

Procollagen *C*-proteinase was purified as described previously (Hojima et al., 1985). One unit was defined as the amount of enzyme that cleaves 1 μg of type I procollagen in 1 h at 35 °C in a reaction system with an initial substrate concentration of 12 μg·mL^{-1}.

Fibril Formation. The pCcollagen substrate and *C*-proteinase were twice dialyzed against 600 volumes of fibril formation buffer (Kadler et al., 1987) that was a modified Dulbecco's medium and consisted of 20 mM $NaHCO_3$, 117 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.81 mM $MgSO_4$, 1.03 mM NaH_2PO_4, and 0.01% NaN_3, pH 7.3 at 20 °C. The buffer was stored at 4 °C under an atmosphere of 10% CO_2 and 90% air without precipitation or change in pH. Procollagen, pCcollagen, and C-proteinase were soluble at high concentrations in the buffer. To initiate fibril formation, pCcollagen and *C*-proteinase solutions were mixed in a microcentrifuge tube at 4 °C, and the tube was charged briefly with water-saturated 10% CO_2 and 90% air. To prevent evaporation, the 1.5-mL microcentrifuge tube was sealed with a shortened plunger from a 3-mL plastic syringe that was held in place by closing the cap on the tube.

For assays of collagen in the soluble and polymer phases, reaction mixtures of 40 μL were incubated at the experimental temperatures and then centrifuged at 15600*g* for 4 min at room temperature. The supernatants were recovered, and the pellets were suspended in 40 μL of fibril formation buffer. The samples were prepared for gel electrophoresis by adding 10 μL of 5 × sample buffer or 40 μL of 2 × sample buffer and heating at 100 °C for 3 min. The 5 × sample buffer consisted of 10% SDS, 50% glycerol, and 0.01% bromphenol blue in 0.625 M Tris-HCl buffer, pH 6.8. The samples were separated by discontinuous SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) using a 7% separating gel and a 3.5% stacking gel. Gels were prepared for fluorography using 20% 2,5-diphenyloxazole in glacial acetic acid and exposing dried gels to preflashed Kodak XAR-5 film at −70 °C. Fluorograms were scanned by using a laser densitometer. The concentrations of collagens were determined by measuring the intensity of α1(I) bands in fluorograms of the pellet and supernatant fractions adjusted for the initial amount in the sample.

Three species of collagen generated from the proband's pCcollagen were assayed as described previously (Vogel et al., 1987, 1988) and in Figure 1. Because of the large differences in the concentrations of collagen in the pellet and supernatant fractions, multiple exposures of gels of 24 h to 6 weeks were used.

To examine fibril assembly by dark-field light microscopy, pCcollagen and *C*-proteinase mixtures were incubated in a chamber constructed from plastic cylinders (5-mm internal diameter × 2 mm) affixed to a microscope slide with cyanoacrylate or epoxy glue (Kadler et al., 1990a,b). The chamber were grease-sealed with a coverglass and placed on a piezoelectric heating stage (Model CO60, Linkam) with a range of temperatures from room temperature to 41 °C. Dark-field light pictures were recorded by a Zeiss microscope on Kodak panatomic X 32 ASA negative film.

Electron Microscopy. Fibrils were recovered by centrifugation for 4 min and resuspended in 5–10 μL of 0.1 M Tris-HCl buffer, pH 7.4 at 20 °C. The fibrils were applied to 200-mesh copper grids coated with a formvar film and pretreated with 100 mg·mL^{-1} poly(DL-lysine). Excess solution was removed from the grids by touching the edges with filter paper. The grids were inverted on a drop of phosphate-buffered saline for 5 min, a drop of periodate–lysine–para-

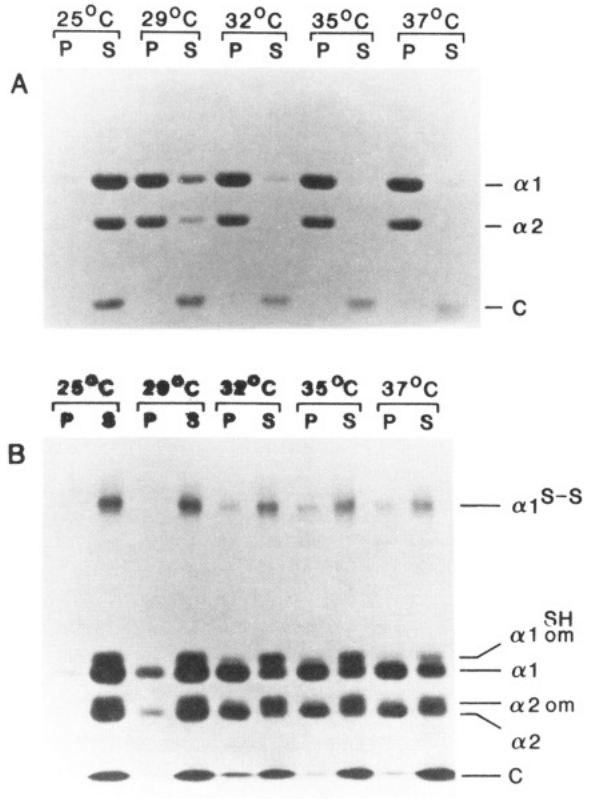


FIGURE 1: Analysis by polyacrylamide gel electrophoresis in SDS of pellet and supernate fractions obtained after cleavage of type I pC-collagen with procollagen *C*-proteinase. The samples contained initial concentrations of 70 $\mu g \cdot mL^{-1}$ type I pCcollagen and 35–145 $units \cdot mL^{-1}$ *C*-proteinase after adjustment for temperature. The samples were incubated at the temperatures indicated for 24 h, and then the pellet and supernate fractions were isolated by a brief centrifugation. (A) Control pCcollagen. (B) Proband's pCcollagen (preparation 1 in Table I). Symbols: P, pellet fraction; S, supernate; α1 and α2, two polypeptide chains of type I collagen; $\alpha 1^{S-S}$, dimers of α1 chains linked through the cysteine at position 748; $\alpha 1^{SH}$, overmodified α1 chains probably containing a cysteine in position 748; α2 om, translationally overmodified α2 chains; C, *C*-propeptide released by cleavage of pCcollagen by *C*-proteinase.

formaldehyde (McLean & Nakane, 1974) for 10 min, a drop of distilled water for 5 min, and a drop of 0.5% uranyl acetate for 5 min. Finally, the grids were rinsed with distilled water for 5 s. The grids were dried and coated with a carbon film and examined in a transmission electron microscope (Model EM 109, Zeiss).

Results

Preparations of the Mutated Type I Procollagens. Collagen generated by cleavage of the proband's type I procollagen with a combination of procollagen *N*-proteinase and *C*-proteinase was comprised of normally migrating α chains and α chains that migrated slowly on SDS gels because of posttranslational overmodification (Figure 1). Under nonreducing conditions, a very slowly migrating band was observed that represented two mutated α1(I) chains linked by a disulfide bond (Vogel et al., 1987, 1988). Therefore, collagen generated from the proband's procollagen was comprised of normal collagen (α1, α1, α2), collagen containing one mutated α1 chain ($\alpha 1^{SH}$, α1, α2), and collagen containing two mutated α1 chains linked by disulfide bonds ($\alpha 1^{S-S}$α1, α2). The proband's collagen probably also contained some molecules with two mutated α1(I) chains that were not disulfide-linked, but such molecules could not be assayed accurately (Vogel et al., 1987, 1988).

For the experiments described here, six different preparations of type I procollagen purified from the medium of the proband's fibroblasts were used (Table I). Analysis of the collagens generated from the procollagens demonstrated that they varied in their contents of normal and mutated protein. The content of normal α1(I) chains varied from 63 to 80% of the total α1(I) chains. The content of overmodified α1(I) chains that were not disulfide-linked varied from 10 to 27%. The content of disulfide-linked α1(I) chains varied from 7 to 18% of the total α1(I) chains. The reasons for the variations were not apparent. For example, the content of mutated α1(I) chains was not related in a simple manner to the passage number of the fibroblasts, the state of confluency of the cells, or the presence of fetal calf serum in the medium. Because of variations in the overall yields, it was not possible to exclude the possibility that the differences among the preparations were explained by differential losses of the mutated and normal proteins during the chromatographic steps or procedures used to concentrate the samples.

Table I: Variation in the Contents of Normal and Abnormal Collagen Generated by Enzymic Cleavage of Different Preparations of the Proband's Type I Procollagen

prepn	α chains (% of total)[a] normal α chains	α chains (% of total)[a] overmodified α chains	disulfide-linked α1(I) chains
1[b]	67	15	18
2[b]	63	21	16
3	80	10	10
4	75	15	10
5	80	13	7
6	65	27	8

[a] Species of normal, overmodified, and disulfide-linked collagens were assayed by polyacrylamide gel electrophoresis in SDS under nonreducing conditions after cleavage of the type I procollagen to collagen with procollagen *N*-proteinase and *C*-proteinase. For preparations 1–5, values were based on densitometry of α1(I) chains (see text and Figure 1). For preparation 6, separation of normal and overmodified chains was adequate to assay also the α2(I) chains, and to establish that the values were similar if based solely on α1(I) chains or on both α1(I) and α2(I) chains. [b] Preparations that gave dendritic fibrils (see Figures 2–4).

Incorporation of the Mutated Collagens into Polymers. To determine whether the mutated collagens were incorporated into large polymers, pCcollagen was prepared from the proband's procollagen and then was cleaved to collagen with *C*-proteinase under the conditions used to generate fibrils de novo (Kadler et al., 1987, 1988). In initial experiments (Figure 1), samples were incubated at 25–37 °C for 24 h. The samples were centrifuged briefly to separate fibrils from the solution phase, and the pellets and supernatants were analyzed by gel electrophoresis in SDS and fluorography. As reported previously (Cooper, 1970; Kadler et al., 1987, 1988; Na et al., 1989), the incorporation of normal collagen into fibrils was markedly dependent on temperature (Figure 1A). A similar temperature dependence was seen with collagen from the proband's fibroblasts (Figure 1B). The pellet fractions contained primarily normal collagen, but some of them also contained disulfide-linked α1(I) chains and overmodified α1(I) chains not disulfide-linked.

Branched Fibrils Formed with the Two Preparations of Mutated Collagen with a High Disulfide Content. Assembly of the collagens was examined by dark-field light microscopy of samples placed in small chambers designed so that it was possible to follow assembly of all the large structures formed (Kadler et al., 1990a).

Initial experiments were carried out at 32 °C, a temperature at which type I collagen from control fibroblasts assembled into fibrils that were relatively thick and needlelike (Kadler et al., 1990a). The length of the fibrils varied somewhat in

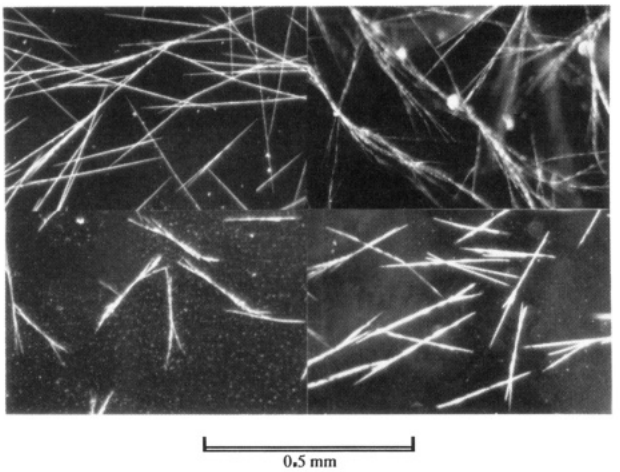


FIGURE 2: Dark-field light microscopy of large structures formed from normal and mutated type I collagen. Samples were prepared by incubating 50 $\mu g \cdot mL^{-1}$ type I pCcollagen with 64 units$\cdot mL^{-1}$ procollagen *C*-proteinase at 32 °C for 24 h. Upper left frame: Control type I pCcollagen. Upper right frame: Preparation 2 (Table I) of the proband's type I pCcollagen. Lower left frame: Same pCcollagen as upper right except diluted 1:1 with normal pCcollagen from control fibroblasts. Lower right frame: preparation 6 of the proband's type I pCcollagen.

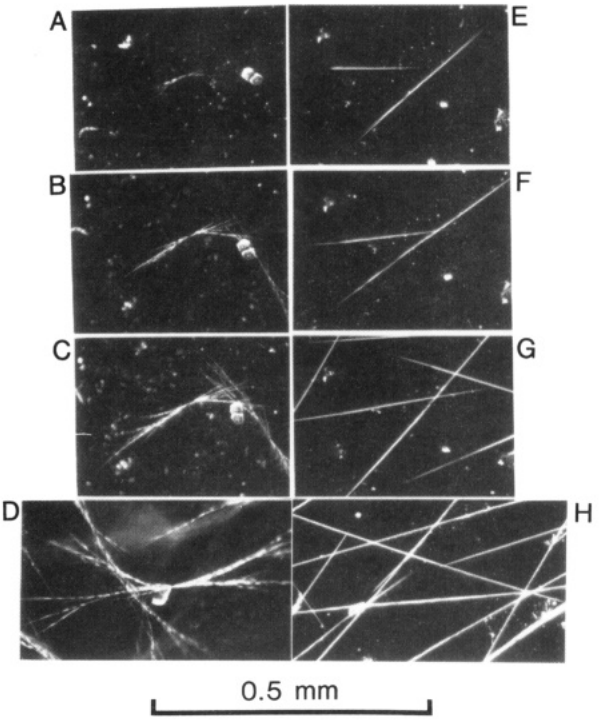


FIGURE 3: Time-lapse dark-field light microscopy of fibril formation. Type I pCcollagen initial concentration of 70 $\mu g \cdot mL^{-1}$ was incubated at 30 °C with 64 units$\cdot mL^{-1}$ of *C*-proteinase. (A–D) Samples containing preparation 2 of the proband's type I pCcollagen photographed at 3, 4, 5, and 24 h, respectively. (E–H) Parallel samples of normal type I pCcollagen from control fibroblasts under the same conditions.

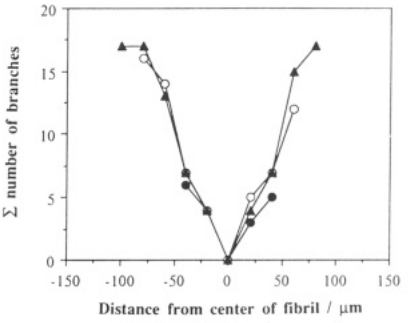


FIGURE 4: Analyses of the structures formed with preparation 2 of proband's type I pCcollagen. Structures similar to those shown in Figure 2 (upper right) and Figure 3 (frames A–D) were analyzed by measuring the number of readily discernible branch points as a function of the distance from the center of the structure. Data from three structures are included in the figure.

that some preparations generated longer fibrils at 32 °C than those shown in Figure 2 (upper left). Also, some of the preparations generated a few fibrils with occasional branch points [see Miyahara et al. (1982, 1984)]. The differences observed among different preparations from the medium of control fibroblasts were probably explained by the presence of small amounts of contaminants that were introduced in processing the samples, or that copurified through four chromatographic steps and then bound to the growing tips of the fibrils (Kadler et al., 1990b).

Preparation of the proband's type I pCcollagen consistently generated fibrils that had a different morphology than type I pCcollagen from control fibroblasts. With preparations 1 and 2 that had a high content of disulfide-linked $\alpha 1$(I) chains (Table I), the fibrils were highly branched and dendritic (Figure 2, upper right). Examination of all of the fibrils in repeated experiments demonstrated that all had the same highly branched appearance, and none of the structures had the needlelike appearance of normal fibrils (Figure 2, upper left). Diluting the pCcollagen prepared from preparation 1 with control pCcollagen so that the concentration of disulfide dimer decreased from 18% to 9% generated fibrils that were no longer branched but were still distinguishable from controls because the fibrils were shorter, thicker, and more blunt (Figure 2, lower left). With a 10:1 mixture of control to mutated pCcollagen, the fibrils were indistinguishable from those formed with control pCcollagen (not shown).

The same experiments were repeated at 37 °C. Because the type I collagen formed fibrils that were longer and thinner at 37 °C than at 32 °C (Kadler et al., 1990a), individual fibrils were more difficult to distinguish clearly than with fibrils generated at lower temperatures. However, the fibrils formed from the mutated type I collagen (preparations 1 and 2) were again readily distinguishable because all were highly branched (not shown).

Formation of Fractallike Structures with the Two Preparations Containing a High Content of Disulfide-Linked Pro$\alpha 1$(I) Chains. The two preparations of type I procollagen with the high disulfide content (preparations 1 and 2 in Table I) were used to study in more detail the assembly of fibrils. Fibrils were generated by incubating 70 $\mu g \cdot mL^{-1}$ pCcollagen with 50 units$\cdot mL^{-1}$ *C*-proteinase at 32 °C. As examined by dark-field microscopy, the first fibrils formed from the proband's collagen were highly branched (Figure 3A). The number of branches then increased as the fibrils grew in size (Figure 3A–D). Therefore, the branching was an integral part of the fibril growth.

The branching of the fibrils formed by the proband's collagen appeared to be symmetrical (Figure 2, upper right, and Figure 3A–D). Therefore, the geometry of the structures was analyzed with the formula $N = L^D$, where N is the number of branches or other discontinuities in the structure, L is the distance from the center of the structure, and the power term D, is the "fractal dimension" (Mandelbrot, 1983). The value of D observed here was 1.6 (Figure 4), the value seen in most fractals (Mandelbrot, 1983).

Thicker Fibrils Formed by Three Preparations of Mutated Collagen with a Low Disulfide Content. Similar fibril experiments were carried out with three preparations of type I

Table II: Diameter of Fibrils Formed from Normal and Mutated Type I Collagen[a]

collagen	incubn time (h)	no. of fibrils measured	mean diameter[b] (nm)	standard deviation
control	1	86	145	±54
	48	0[c]		
proband	1	166	299[d]	±196
	48	70	650	±130

[a] Fibrils were formed at 37 °C by cleavage of 200 $\mu g \cdot mL^{-1}$ pC-collagen with 50 units·mL^{-1} *C*-proteinase. Samples were centrifuged at 1 h, and the pellets were recovered for analysis by electron microscopy. The supernates were incubated at 37 °C for an additional 47 h, and then a second pellet fraction was recovered for electron microscopy. The pCcollagen was preparation 6 (Table I). [b] Widest diameter observed for a given fibril. [c] No fibrils seen on grids. [d] Individual fibrils appeared to be aggregated and flattened.

pCcollagen that had lower contents of disulfide-linked proα1(I) chains, i.e., 7–10% instead of 16 or 18% (Table I). The fibrils generated at 32 °C from preparations 3, 4, and 6 appeared to be thicker and more rigid as seen by dark-field light microscopy (Figure 2, lower right) than those formed from control type I pCcollagen under the same conditions (Figure 2, upper left). Also, the pointed tips were less apparent, and both ends appeared blunt. The fibrils were similar in appearance to the fibrils formed by diluting pCcollagen from preparation 1 with control pCcollagen to adjust the proportion of disulfide-linked dimer to 9% (compare Figure 2, lower left and lower right). Similar results were obtained with fibrils formed at 37 °C (not shown).

The fibrils formed from preparation 6 were also examined by transmission electron microscopy after being stained with uranyl acetate (Table II). In the first series of experiments, type I pCcollagens from control fibroblasts and the proband's fibroblasts were incubated at 37 °C with 500 units·mL^{-1} *C*-proteinase and an initial substrate concentration of 200 $\mu g \cdot mL^{-1}$. After 48 h, the samples were centrifuged, and the pellets were prepared for microscopy. The fibrils generated from control samples were similar to those observed previously (Kadler et al., 1990b). In contrast, two types of fibrils were found in samples of preparation 6 (not shown). One type was indistinguishable from control fibrils. The others were thicker and straighter. Both types of fibrils showed a characteristic D-periodic banding pattern. Of special note was that the tips of the thicker fibrils were less tapered and more blunt than the tips of control fibrils.

The time course experiments (see below) suggested that most of the normal collagen generated from the proband's pCcollagen was incorporated into fibrils during the first few hours when the experiments were carried out at 37 °C. Therefore, to separate the two kinds of fibrils, an experiment was performed in which fibrils formed after incubation at 37 °C for 1 h were removed from the samples by brief centrifugation. The supernatants were then incubated at 37 °C for an additional 47 h. Most of the fibrils collected after 1 h were thin, flexible, and similar to control fibrils (not shown). Some of the fibrils tended to aggregate. Therefore, the maximal mean diameter and standard deviation of measured fibrils were both larger than the control (Table II). All the fibrils recovered after the second incubation were thick. The maximal mean diameter at the thickest part of the fibrils was 4 times the mean diameter of control fibrils (see Table II). Also, all the tips appeared to be more blunt than the tips of control fibrils (not shown).

Delayed Incorporation of Normal Collagen. The rates of polymerization of normal and mutated collagens in a preparation of the proband's protein (preparation 6 in Table I) were

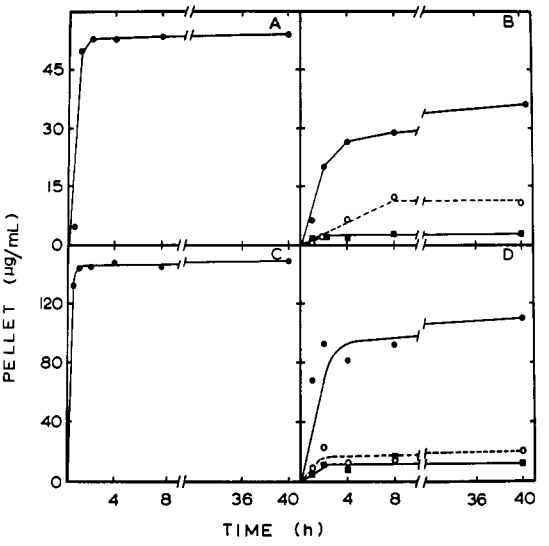


FIGURE 5: Incorporation of normal and mutated type I collagens into large structures at 37 °C. Each sample contained either 70 or 200 $\mu g \cdot mL^{-1}$ type I pCcollagen and was incubated with 50 units of partially purified *C*-proteinase for the times indicated. Supernate and pellet fractions were isolated by brief centrifugation and analyzed as shown in Figure 1. (A) Control type I pCcollagen from normal fibroblasts, initial substrate concentration 70 $\mu g \cdot mL^{-1}$; (B) preparation 6 (Table I) of type I pCcollagen from the proband's fibroblasts, initial substrate concentration 70 $\mu g \cdot mL^{-1}$; (C) normal type I pCcollagen from control fibroblasts, initial substrate concentration 200 $\mu g \cdot mL^{-1}$; (D) preparation 6 of type I pCcollagen from proband's fibroblasts, initial substrate concentration 200 $\mu g \cdot mL^{-1}$. Symbols: (●) normal type I collagen; (○) collagen comprised of overmodified α1(I) chains; (■) collagen containing disulfide-linked dimers.

Table III: Initial Rates of Polymerization of Normal and Mutated Collagens[a]

temp (°C)	sample	initial concn ($\mu g \cdot mL^{-1}$) total pCcollagen	initial concn ($\mu g \cdot mL^{-1}$) normal pCcollagen	initial rate of polymerization[b] ($\mu g \cdot h^{-1}$)
37	control procollagen	70	70	40
		200	200	280
	proband's procollagen	70	45	10
		200	130	30
32	control procollagen	70	70	25
		200	200	140
	proband's procollagen	70	45	<2
		200	130	20

[a] Experiments were carried out with preparation 6 (Table I) and under the conditions indicated in Figure 5. [b] Values are for initial rates of polymerization of normal collagen only.

examined at two different temperatures and substrate concentrations. To facilitate analysis of the data, high concentrations of procollagen *C*-proteinase were used to that all the pCcollagen was cleaved to collagen in less than 30 min (not shown).

The first series of experiments were performed at 37 °C (Figure 5). With an initial substrate concentration of 70 $\mu g \cdot mL^{-1}$, the initial rate of polymerization of normal collagen from control fibroblasts was about 40 $\mu g \cdot h^{-1}$ (Figure 5A and Table III). Under the same conditions, the initial rate of polymerization of normal collagen from the mixture of normal and mutation collagen found in a preparation of the proband's protein was about 10 $\mu g \cdot h^{-1}$ (Figure 5B). With an initial substrate concentration of 200 $\mu g \cdot mL^{-1}$, the initial rate of polymerization of normal collagen from control fibroblasts was about 280 $\mu g \cdot h^{-1}$ (Figure 5C). Under the same conditions, the initial rate of polymerization of normal collagen from a

Table IV: Equilibrium Concentrations of Normal and Mutated Collagens[a]

temp (°C)	initial concn ($\mu g \cdot mL^{-1}$)	time (h)	control collagen ($\mu g \cdot mL^{-1}$)	proband's collagen ($\mu g \cdot mL^{-1}$)			
				total	normal	overmodified	disulfide-linked
37	70	40	0.3	4.7	1.0	2.6	1.1
	200	40	0.9	13.0	1.0	9.0	3.0
32	70	24	1.5	13.8	4.7	7.4	1.7
	70	45	1.1	15.1	2.2	9.7	3.2
	70	72	3.0	16.2	14.2[b]		2.1
	200	24	2.0	31.5	1.5	24.0	6.0
	200	48	3.0	37.5	4.5	25.5	7.5

[a] Experiments were carried out with preparation 6 (Table I) and under the conditions indicated in Figure 5. The collagens were assayed by polyacrylamide gel electrophoresis in SDS under nonreducing conditions. The relative amounts of each species of collagen in the supernatants were assayed by densitometry of the normal $\alpha 1$(I) and $\alpha 2$(I) chains, the overmodified $\alpha 1$(I) and $\alpha 2$(I) chains, and the disulfide-linked $\alpha 1$(I) chains in the supernatant and pellet fractions. The values were then calculated as micrograms per milliliter on the basis of the initial cpm of ^{14}C-labeled pC-collagen added and the specific activity of the protein. [b] Bands of normal and overmodified α chains were not adequately resolved on fluorograms of SDS–polyacrylamide gels.

preparation of the proband's protein was about 30 $\mu g \cdot h^{-1}$ (Figure 5D).

Similar differences between normal collagen and the proband's collagen were observed at 32 °C (Table III).

The effects of the mutated monomers were not explained by lower concentrations of normal monomers in preparation of the proband's pCcollagen. As indicated in Table III, an initial concentration of the proband's pCcollagen of 200 $\mu g \cdot mL^{-1}$ contained 130 $\mu g \cdot mL^{-1}$ normal pCcollagen. However, the initial rate of polymerization was slightly less than the initial rate of polymerization observed with just 70 $\mu g \cdot mL^{-1}$ control pCcollagen.

Equilibrium Concentrations of Collagens. Under the conditions employed here, the system reached equilibrium in 4–48 h, depending on the temperature, initial concentration of pCcollagen, and concentration of *C*-proteinase (Figure 5 and Table IV).

With collagen from the proband's fibroblasts, the solution-phase concentration of total collagen in equilibrium with fibrils was consistently 5–10 times the value observed with normal collagen from control fibroblasts (Table IV). Also, higher values were obtained with the higher initial substrate concentration. At 37 °C, the value was 4.7 $\mu g \cdot mL^{-1}$ with an initial substrate concentration of 70 $\mu g \cdot mL^{-1}$. It was 13.0 $\mu g \cdot mL^{-1}$ with an initial substrate concentration of 200 $\mu g \cdot mL^{-1}$. At 32 °C, the value was 13.8–16.2 $\mu g \cdot mL^{-1}$ with an initial concentration of 70 $\mu g \cdot mL^{-1}$. It was 31.7–37.5 $\mu g \cdot mL^{-1}$ with an initial concentration of 200 $\mu g \cdot mL^{-1}$. The major effect was the high equilibrium concentration of overmodified $\alpha 1$(I) chains that were not disulfide-linked.

Discussion

A number of mutations in type I procollagen genes that cuase lethal variants of osteogenesis imperfecta are dominant (Prockop et al., 1988; Byers et al., 1990; Kuivaniemi et al., 1991). All of them involve synthesis of a structurally abnormal pro$\alpha 1$(I) or pro$\alpha 2$(I) chain. Previous observations suggested that the dominant phenotype was in part explained by the phenomenon of "procollagen suicide" (Williams & Prockop, 1983; Prockop et al., 1988) whereby structurally abnormal proα chains became disulfide-linked at their *C*-propeptide domains to normal proα chains and caused the degradation of both the normal and abnormal chains. With lethal variants containing substitutions of cysteine for glycine residues, however, only part of the procollagen molecules containing mutated proα chains were degraded (Steinmann et al., 1984; Vogel et al., 1987). Therefore, there was no simple explanation as to why the mutations produced lethal phenotypes. The results here establish that a cysteine for glycine substitution that was previously shown to introduce a flexible kink into the pro-

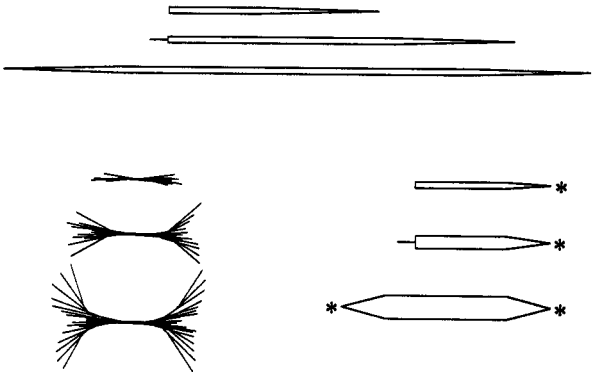


FIGURE 6: Schematic representation of growth of type I collagen fibrils. Top: Growth of normal collagen fibrils [see Kadler et al. (1990b)]. Lower left: Growth of highly branched or dendritic fibrils with two preparations of proband's protein that had a high content of disulfide-linked $\alpha 1$(I) chains (see Figure 2, upper right, and Figure 3A–D). Lower right: Growth of thicker and more blunt fibrils seen with preparations of the proband's protein with a lower content of disulfide-linked $\alpha 1$(I) chains (See Figure 2, lower right, and Table II).

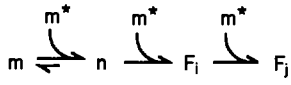
collagen molecule (Vogel et al., 1987, 1988) also has drastic consequences for fibril assembly.

The assembly of collagen into fibrils is a spontaneous process that shows a lag phase (Bensusan & Hoyt, 1958; Gross & Kirk, 1958; Wood & Keech, 1960; Cooper, 1970; Comper & Veis, 1977; Williams et al., 1978; Bernengo et al., 1983; Wallace, 1985; Payne et al., 1986), a critical concentration for polymerization (Kadler et al., 1987; Na et al., 1989), and other features that are characteristic of a cooperative nucleation–growth system for protein self-assembly (Figure 6). Therefore, assembly of collagen into fibrils is similar to the assembly of actin into filaments (Frieden & Goddette, 1983; Pllard & Cooper, 1986; Frieden, 1989), the assembly of tubulin into microtubules (Timasheff & Grisham, 1980; Mitchison & Kirschner, 1984; Olmsted, 1986), and the polymerization of HbS into filaments (Hofrichter, 1986; Noguchi & Schecter, 1985; Adachi et al., 1987). Previous observations with the fibroblasts studied here (Vogel et al., 1987, 1988) demonstrated that the cells synthesized and secreted a mixture of both normal type I procollagen and mutated type I procollagen containing a cysteine substitution for glycine $\alpha 1$–748. It was not apparent, however, whether the mutated procollagen secreted by the proband's fibroblasts was a precursor of a collagen monomer that could polymerize into fibrils. As reviewed by Oosawa and Asakura (1975), two or more monomers in the same solution that have very different affinities will not copolymerize into the same structures. For example, HbS readily polymerizes into filaments, but under most experi-

mental conditions, the filaments formed from solutions containing mixtures of HbS and HbA contain HbS but not HbA (Adachi et al., 1987). If two or more monomers have similar affinities, they can form copolymers (Oosawa & Asakura, 1975). The results here establish that the normal and mutated collagens generated from the proband's procollagens are similar enough in their affinities to copolymerize.

The conclusion that the normal and mutated collagens formed copolymers was based on two independent observations. One came from the experiments in which the morphology of the fibrils was examined. With the two preparations with a high content of disulfide-linked $\alpha 1$(I) chains, all the structures seen by dark-field light microscopy had a distinctive, highly branched morphology (Figure 2, upper right, and Figure 3A–D). The same large structures recovered after brief centrifugation of the samples contained both normal and mutated collagens (Figure 1B). Therefore, it was apparent that the highly branched fibrils were true copolymers of normal and mutated collagens. Less dramatic but similar results were obtained with one preparation with a low content of disulfide-linked $\alpha 1$(I) chain in that essentially all the fibrils formed between 1 and 48 h of incubation were thicker than control fibrils (Table II). Similar morphologic criteria were used to establish copolymerization of "normal" and "curly" flagella of salmonella (Asakura et al., 1964; Oosawa & Asakura, 1975). The second observation establishing copolymerization came from the time course studies that established that the presence of mutated collagen in samples of the proband's protein decreased the initial rate of polymerization of the normal collagen in the same samples (Figure 5 and Table III). The decrease in the initial rate was not explained by the lower concentration of normal monomer in the preparation of the proband's protein (Table III). Therefore, it was apparent that under the conditions of these experiments the bulk of the normal collagen in the samples of the proband's protein was copolymerizing with the mutated monomer and that it was not assembling into a separate population of collagen fibrils or being nonspecifically trapped during the assembly of the normal monomers. Similar inhibitory effects of one monomer on polymerization of a second monomer were seen with mixtures of flagellins that copolymerized (Oosawa & Asakura, 1975).

The results here can be interpreted in terms of a modified scheme for polymerization of nucleation and propagation:



in which m is the normal monomer, m* is the mutated monomer, n is the nucleus for propagation of the fibrillar structure, F_i is the initial fibril formed in the system, and F_j is the final fibril seen at equilibrium. Since both the morphology and composition of the fibrils varied under different experimental conditions, it was apparent that the copolymers were heterogeneous and without a fixed stoichiometry of normal and mutated monomers. The heterogeneity is probably explained by the fact that under varying conditions the mutated monomers are incorporated early or late in the process of fibril assembly. Early incorporation was illustrated by the highly branched structures formed from the two preparations with a high content of disulfide-linked $\alpha 1$(I) chains. The results, in fact, suggested that some of the mutated monomers were incorporated into the nucleus, since the highly branched fibrils met one of the formal requirements of a symmetrical fractal (Mandelbrot, 1983). The structure of a nucleus of a polymerization process is difficult to define because it usually consists of two to four monomers and is a transient intermediate in polymerization [see Oosawa and Asakura (1975), Timasheff and Grisham (1980), Pollard and Cooper (1986), and Frieden (1989)]. Formation of an abnormal nucleus, however, is the simplest explanation for fractal geometry of the structures and their symmetrical growth. Incorporation of mutated monomers late in fibril assembly was illustrated by several of the time course experiments in which the overmodified species continued to be incorporated over long periods of time (see Figure 5). The short and blunt fibrils can be explained by binding of mutated monomers to growing tips (Kadler et al., 1990b) so as to interfere with further growth of the tips but not lateral growth of the shaft after initial fibrils (F_i) are assembled from normal monomers.

Copolymerization of the mutated collagen with normal collagen synthesized by the same fibroblasts in vivo may well be a reason why the cysteine $\alpha 1$–748 mutation produced a lethal phenotype that was heterozygous dominant (Vogel et al., 1987, 1988). Similar effects may also occur with the large series of mutations now known that cause osteogenesis imperfecta and change the primary structure of type I procollagen (Prockop et al., 1988; Byers, 1990; Kuivaniemi et al., 1991). However, some substitutions of amino acids with bulkier side chains for glycine residues are lethal whereas others are not. Also, different glycine substitutions found in different variants of osteogenesis imperfecta were shown to have very different effects on the thermal stability and microunfolding of the triple helix (Prockop et al., 1989; Pack et al., 1989; Westerhausen et al., 1990). Therefore, some may form copolymers with normal collagen, and others may not.

Registry No. Glycine, 56-40-6; cysteine, 52-90-4.

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