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**Research Article**

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# A Lethal Variant of Osteogenesis Imperfecta Has a Single Base Mutation That Substitutes Cysteine for Glycine 904 of the α1(I) Chain of Type I Procollagen

## The Asymptomatic Mother Has an Unidentified Mutation Producing an Overmodified and Unstable Type I Procollagen

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## Abstract

**A fraction of the proα1(I) and proα2(I) chains in type I procollagen synthesized by the fibroblasts from a proband with a lethal variant of osteogenesis imperfecta were overmodified by posttranslational reactions. After digestion with pepsin, some of the α1(I) chains were recovered as disulfide-linked dimers. Mapping of cyanogen bromide peptides indicated that the disulfide link was contained in α1-CB6, the cyanogen bromide fragment containing amino acid residues 823–1014 of the α1(I) chain. Nucleotide sequencing of cDNA clones demonstrated a substitution of T for G that converted glycine 904 of the α1(I) chain to cysteine.**

**A large fraction of the type I procollagen synthesized by the proband's fibroblasts had a thermostability that was 3–4°C lower than the normal type I procollagen as assayed by brief proteinase digestion. In addition, the type I procollagen synthesized by the proband's fibroblasts was secreted with an abnormal kinetic pattern in that there was a lag period of about 30 min in pulse-chase experiments.**

**The mutation of glycine to cysteine was not found in type I procollagen synthesized by fibroblasts from the proband's parents. Therefore, the mutation was a sporadic one. However, the mother's fibroblasts synthesized a type I procollagen in which part of the proα chains were overmodified and had a lower thermostability. Therefore, the proband may have inherited a mutated allele for type I procollagen from her mother that contributed to the lethal phenotype. The mother was asymptomatic. She was somewhat short and had slightly blue sclerae but no definitive signs of a connective tissue abnormality. The observations on the mother indicated, therefore, that a mutation that causes synthesis of a type I procollagen with a lowered thermal stability does not necessarily produce a heritable disorder of connective tissue.**

## Introduction

Defects in the synthesis or structure of type I collagen, or its precursor type I procollagen, are found in several heritable disorders of connective tissue. The heritable disorders are heterogenous in their phenotypes but are generally classified as Ehlers-Danlos syndrome (EDS),[1] in which the hallmark is loose joints, or osteogenesis imperfecta (OI), in which the characteristic feature is brittle bones (for reviews, see references 1–4).

Eight probands with EDS or OI had mutations that caused in-phase deletions of amino acid sequences in either the proα1(I) or proα2(I) chain of type I procollagen (5–18). One proband with moderately severe OI had a homozygous mutation that deleted 4 bp of coding sequence and thereby altered the sequence of the last 33 amino acids in the C-propeptide of the proα2(I) chain (19–21). Of special interest are three lethal variants of OI in which there were amino acid substitutions for single glycine residues in the proα1(I) chain. One of the mutations converted glycine in amino acid position 391[2] of the α1(I) chain to arginine (22), another converted glycine in position 748 to cysteine (23), and still another converted glycine in position 988 to cysteine (24, 25). All three mutations produced posttranslational overmodification of the protein, and reduced its rate of secretion from fibroblasts. With the two mutations in which helical stability was examined (23–25), the melting temperature of the type I procollagen was lowered by 2–4°C. The results suggested therefore that any mutation that introduces a bulkier amino acid for glycine into the triple-helical domain of type I procollagen alters the physical and biological properties of the protein sufficiently to produce a lethal phenotype. However, observations on several other probands with OI are not totally consistent with this conclusion. Mutations introducing cysteine residues in the α1(I) chain were found in two probands with moderately severe OI (26, 27). One of the mutations lowered the thermal stability of the protein (26) and the other did not (27). The amino acids replaced by cysteine were not identified, and it was suggested (23, 26, 27) that the cysteine residues did not replace glycine residues but instead replaced amino acids in the X- or Y-position of the repeating -Gly-X-Y- sequence of the α1(I) chain. Another proband with moderately severe OI, however, was recently shown to have a single base mutation that substituted arginine for glycine in position 1012 of the α2(I) chain, the last triplet of

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1. *Abbreviations used in this paper:* CNBr, cyanogen bromide; EDS, Ehlers-Danlos syndrome; OI, osteogenesis imperfecta; $T_m$, midpoint temperature for the helix-to-coil transition of collagen or procollagen.
2. Amino acid positions are numbered by the standard convention in which the first glycine of the triple-helical domain of an α chain is No. 1. The numbers for α1(I) chains can be converted to positions in the human proα1(I) chain by adding 156, and numbers for the α2(I) chain can be converted to the human proα2(I) chain by adding 68.

the triple-helical domain (28). The procollagen was posttranslationally overmodified but its thermal stability was normal.

Here we describe a lethal variant of OI in which a single base mutation converted glycine at position 904 of the $\alpha$1(I) chain to cysteine. In addition, the proband may have inherited a second mutation from her asymptomatic mother that produced an overmodified and thermally unstable species of type I procollagen.

## Methods

*The proband and her family.* The proband (RMS-35) was a female that was stillborn after 31 wk of gestation. She was from the first pregnancy of apparently normal parents who subsequently had two normal children. The proband showed multiple fractures of long bones and ribs. The skull was not ossified and as a result was membranous and very soft. The mother was 26 yr old at the time of the pregnancy. At age 35 she was asymptomatic as was her 45-yr-old husband. Pictures of the mother as a child showed the triangular facies and frontal bossing frequently seen in patients with OI. When examined at age 35, her only notable features were slightly blue sclerae and mild frontal bossing. She was 153 cm tall; her mother was 160 cm and her father 170 cm. The maternal grandmother, who was 63 yr old, had a history of mild psoriasis and polyarthritis. She had Heberden's nodes, Bouchard nodes, and other signs of mild osteoarthritis of the hands together with a mild psoriatic rash. There were no other positive findings on examination of the father or the mother's father, her brother, and her two living children. No member of the family had arcus senilis, apparent hearing loss, tooth abnormalities, or history of fractures. The bone status of the mother and grandmother was evaluated by Drs. Bente Juel Riis and Claus Christiansen, Glostrup Hospital, Glostrup, Denmark. Both had normal values for serum electrolytes, albumin, creatinine, alkaline phosphatase, and osteocalcin. Photon densitometry gave normal values for bone mineral density of the spine, and for bone mineral content of spine and ultradistal forearm. The bone mineral content of the midshaft forearm was in the lower range of normal for both women. The value for the mother was 34.9 U vs. an age-corrected normal range of 32.0–48.9. The value for the grandmother was 23.9 U vs. a normal range of 23.1–43.5. Skin fibroblasts were cultured from the proband, the mother, and the father. To insure that the proband, maternal, and paternal cells were correctly identified, Southern blot analysis was performed using a DNA probe for a hypervariable locus on the X chromosome (29). All the bands seen with the proband's DNA after digestion with TaqI were accounted for by bands seen in the mother's or father's DNA. Therefore, the results were consistent with the initial identification of the cell lines.

The control line of human skin fibroblasts (GM 3349) was obtained from the Human Genetic Mutant Cell Repository (Camden, NJ).

*Cell culturing and labeling.* Cells from passages 6–14 were grown in 25-cm$^2$ plastic flasks (Falcon Labware, Oxnard, CA) in Dulbecco's modified Eagle's medium with 10% fetal calf serum (6). At confluency, the cells were incubated for 4 h in fresh medium containing 55 $\mu$g/ml ascorbate and 50 $\mu$Ci/ml L-[2,3,4,5$^3$H]proline (102 Ci/mmol; Amersham Corp., Arlington Heights, IL). In experiments in which 0.3 mM $\alpha,\alpha'$-dipyridyl was added to inhibit posttranslational reactions, the ascorbate was omitted. At the end of labeling period, the medium was

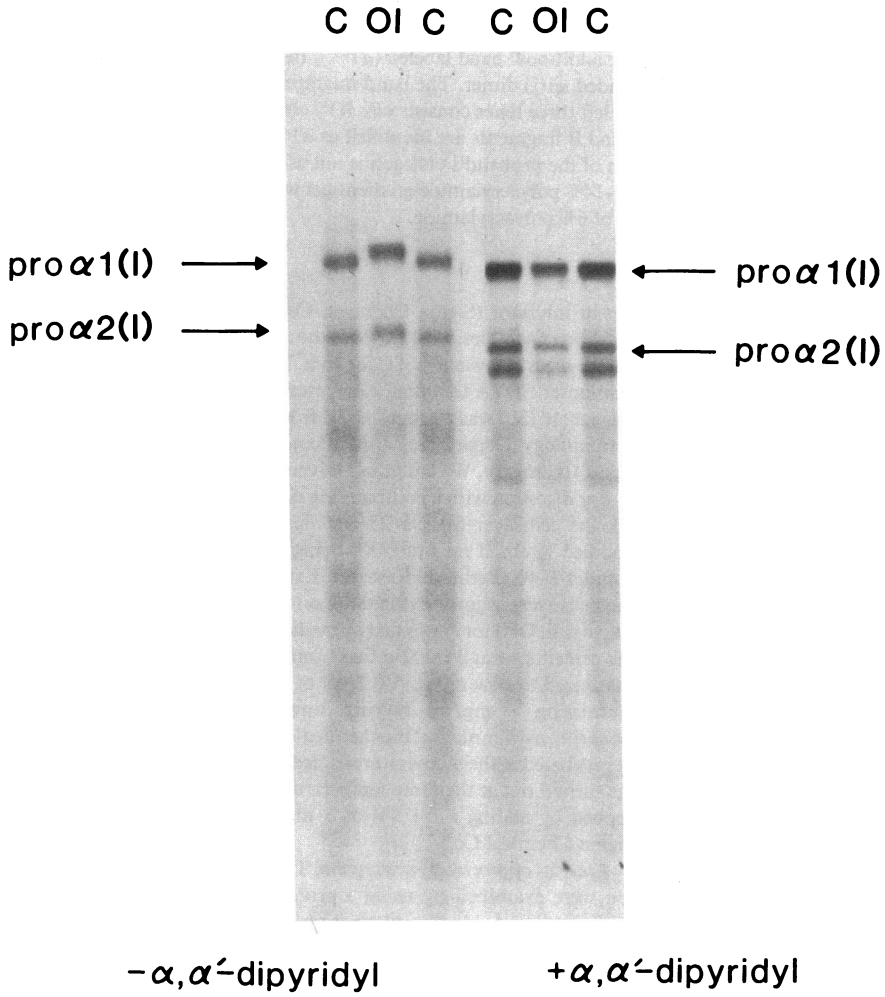


*Figure 1.* SDS-polyacrylamide gel electrophoresis of proteins from control and patient's fibroblasts. Cells were cultured and labeled with [$^3$H]proline for 4 h either with (*right*) or without (*left*) $\alpha,\alpha'$-dipyridyl. After homogenization and denaturation with boiling in the presence of SDS, the samples were reduced with 2-mercaptoethanol and aliquots were electrophoresed on a 6% polyacrylamide gel. *C*, control samples; *OI*, samples from the proband's fibroblasts. The band migrating just below pro$\alpha$2(I) chains in some of the samples is an unidentified protein that was not consistently observed.

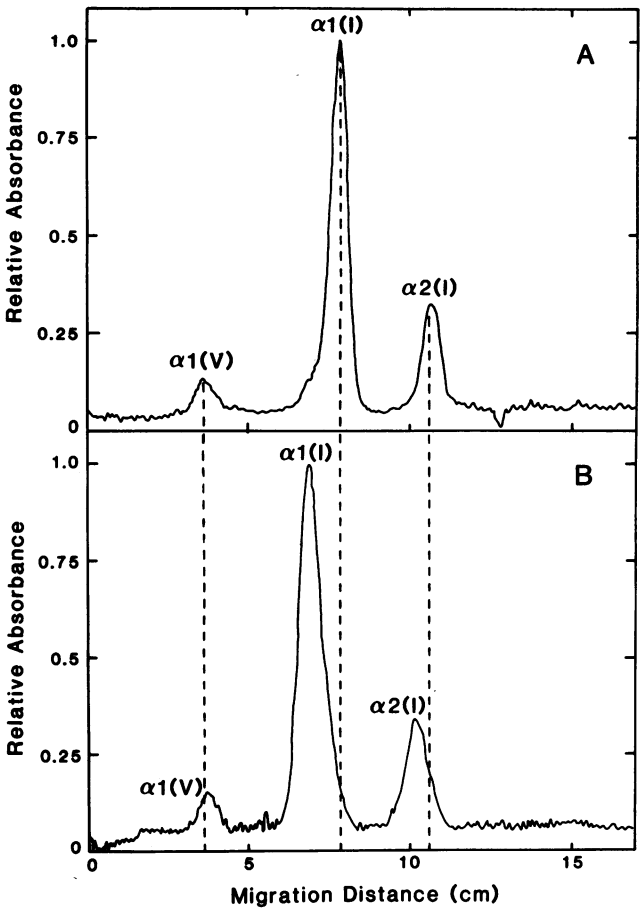


*Figure 2.* Relative migration of α chains from pepsinized procollagens. Fibroblasts were labeled with [$^3$H]proline and homogenates were digested with pepsin as described in text. The samples were separated by electrophoresis on 6% polyacrylamide gels and fluorograms were analyzed by densitometry. (*A*) Sample from control fibroblasts. (*B*) Sample from proband's fibroblasts.

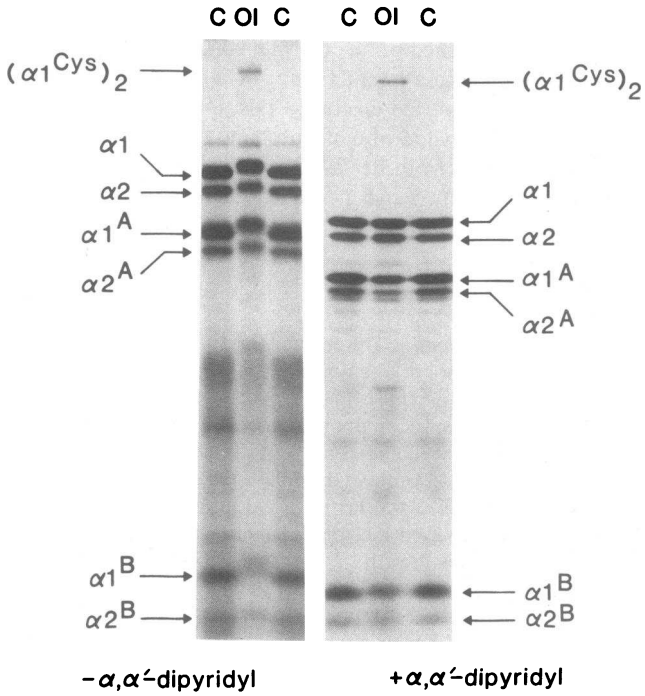


*Figure 3.* SDS-polyacrylamide gel electrophoresis of pepsin- and vertebrate collagenase-treated proteins from control (*C*) and patient's (*OI*) fibroblasts. Cell layer labeled samples were digested with pepsin to remove the N- and C-propeptides. Subsequently, the samples were partially digested with vertebrate collagenase and electrophoresed on a 6–14% gradient SDS-polyacrylamide gel, without prior reduction. The additional band labeled $(\alpha1^{Cys})_2$ in the OI samples is a disulfide-bonded α1(I) dimer. The band migrating just above α1(I) chains in the left three lanes consists of α1(V) chains (42, 43). The collagenase A and B fragments are identified as $\alpha1^A$, $\alpha1^B$, etc. The overmodification of the proband's collagen is not as apparent as in Fig. 1 because a 6–14% polyacrylamide gradient gel was used instead of a uniform gel of 6% polyacrylamide.

removed and the cell layer was washed three times with 2 ml of cold phosphate-buffered saline.

*Analysis of newly synthesized proteins.* Newly synthesized proteins were analyzed by SDS-polyacrylamide gel electrophoresis before and after a series of proteolytic digestions.

For digestion of the cell-layer proteins with pepsin, the cell layer from a 25-cm$^2$ flask was scraped into 0.5 ml of homogenizing buffer in 0.1 M Tris/HCl buffer (pH 7.4) that contained 0.4 M NaCl, 55 mM sodium EDTA, 22 mM *N*-ethylmaleimide, 1.8 mM *p*-aminobenzamidine, 2.2 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100, and 0.2 mg/ml heat-denatured type I collagen (calf skin; Sigma Chemical Co., St. Louis, MO). The sample was homogenized with 10–15 strokes of a motor-driven Teflon and glass homogenizer at 4°C. An equal volume of 0.2 M acetic acid was added, and the pH adjusted to 2–3 with 6 M HCl. A 0.1 vol of a stock pepsin (Boehringer-Mannheim Biochemicals, Indianapolis, IN) solution of 1 mg/ml in 0.1 M acetic acid was added, and the sample was incubated at 18°C for 2 h or at 4°C for 15 h. The sample was neutralized by raising the pH to 8 with 5 M NaOH and left at 4°C overnight.

For samples that were subsequently digested with vertebrate collagenase (30), the pepsinized procollagens were dialyzed extensively at 4°C against 10 mM $CaCl_2$ in 50 mM Tris/HCl buffer adjusted to pH 7.5 at room temperature. Vertebrate collagenase in a concentration of 100–150 μg/ml was activated by adding a 0.1 vol of 0.5 mg/ml trypsin in 10 mM $CaCl_2$/50 mM Tris-HCl (pH 7.5), incubating for 10 min at room temperature, and then adding a 0.1 vol of 2 mg/ml soybean trypsin inhibitor (Sigma Chemical Co.) in the same buffer. For the digestion of samples, a 0.125 vol of the activated collagenase was added and the digestion was at 18°C for 15 h. The reaction was stopped by the addition of EDTA to a final concentration of 15 mM. The vertebrate collagenase (30) was a generous gift from Dr. John Jeffrey, Division of Dermatology, Department of Medicine, School of Medicine and Biological Chemistry, Washington University, St. Louis, MO.

For digestion with a combination of trypsin and chymotrypsin (31, 32), the cell layer from a 25-cm$^2$ flask was scraped into 0.5 ml of modified Krebs II medium (33) containing 10 mM EDTA and 0.1% Nonidet P-40 (Bethesda Research Laboratories, Gaithersburg, MD). The cells were vigorously agitated on a Vortex mixer (Vortec Corp., Cincinnati, OH) for 1 min and immediately cooled to 4°C. The sample was centrifuged at 16,000 *g* for 3 min at 4°C. The supernatant was transferred to new tubes. A 0.1 vol of the modified Krebs II medium containing 1 mg/ml trypsin and 2.5 mg/ml chymotrypsin (Boehringer-Mannheim Biochemicals) was added. The sample was preincubated at the temperature indicated for 10 min and the digestion was carried out at the same temperature for 2 min. The digestion was stopped by adding a 0.1 vol of 5 mg/ml soybean trypsin inhibitor (Sigma Chemical Co.).

*Kinetics of procollagen secretion.* The kinetics of procollagen secretion were examined by using a protocol previously employed (34). Briefly, cultured skin fibroblasts were labeled with [$^3$H]proline for 1–4 h and then the label was chased for up to 24 h by replacing the medium with fresh serum-free medium containing 10 mM unlabeled proline.

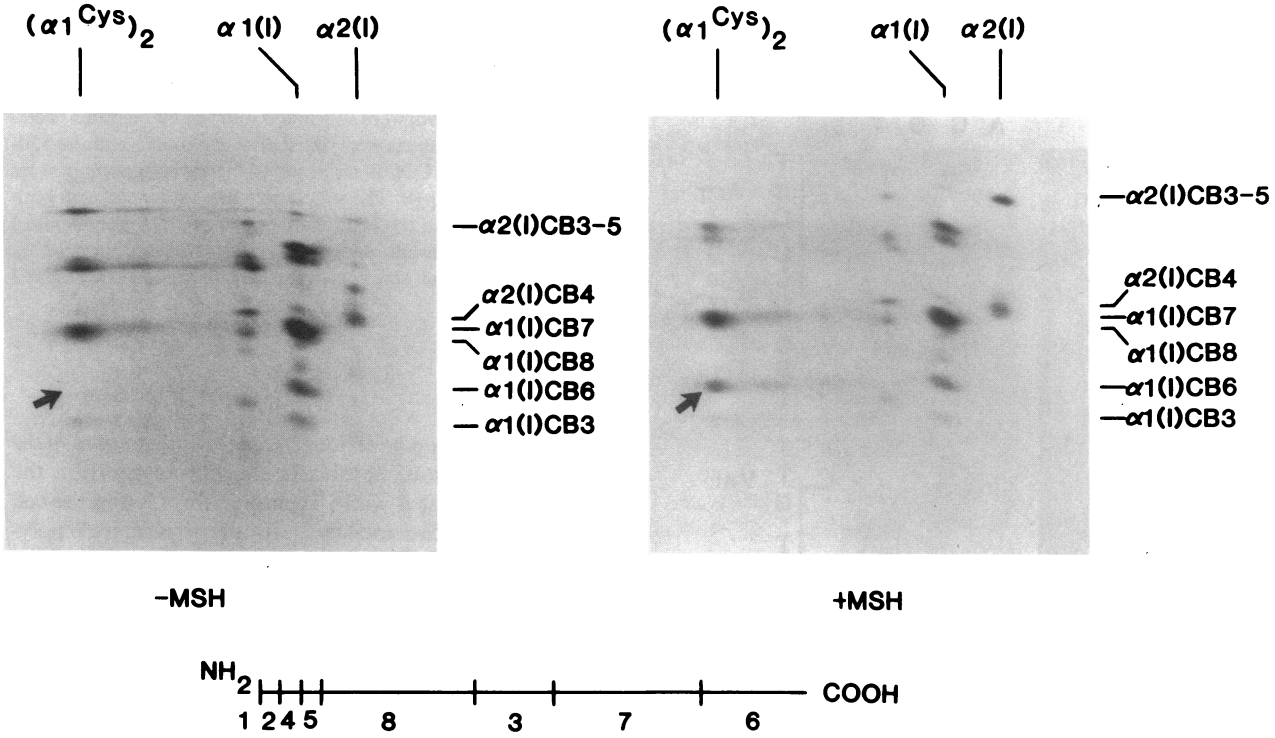


*Figure 4.* Two-dimensional mapping of CNBr peptides from collagens synthesized by the patient's fibroblasts. Pepsin-treated procollagens synthesized by the OI fibroblasts were electrophoresed in one dimension on a 5% SDS-polyacrylamide gel without reduction. Individual lanes were excised from the gel and treated with CNBr in 70% formic acid. The gel strips were then placed horizontally on top of a second gel, and the peptide fragments were separated through a 12.5% separating and 8% stacking gel, with (+*MSH*) or without (−*MSH*) prior reduction with 2-mercaptoethanol. In the *left panel* (no reduction) all CNBr fragments of the α1(I) chain are present in the lane generated from the dimer except for α1-CB6. Upon reduction (*right*), the α1-CB6 is present (*arrows*), an observation indicating that the dimer consists of two α1 chains that are linked by a disulfide bond involving α1-CB6 fragments. The bottom of the figure shows the arrangement of the CNBr fragments in the α1(I) chain.

The cell layer from 25-cm$^2$ flasks was scraped into 0.5 ml of modified Krebs II medium containing 10 mM EDTA and 0.1% Nonidet P-40, and digested with trypsin-chymotrypsin at room temperature as described above. The media proteins were precipitated with 176 mg/ml ammonium sulfate and centrifuged at 10,000 *g* for 1 h at 4°C. The pellets were solubilized and digested with trypsin-chymotrypsin at room temperature. Samples were separated by electrophoresis (see below) and several exposures of gel fluorograms from two separate experiments were scanned with an Ultrascan XL laser densitometer (LKB Instruments, Inc., Gaithersburg, MD). The data were examined on a semilog plot and the rate constants of secretion were calculated from the relationship, $T = Ae^{-k_1 t} + Be^{-k_2 t}$, where $T$ is the total intracellular procollagen, $A$ and $B$ are constants, $k_1$ and $k_2$ are first-order rate constants, and $t$ is time in minutes (34).

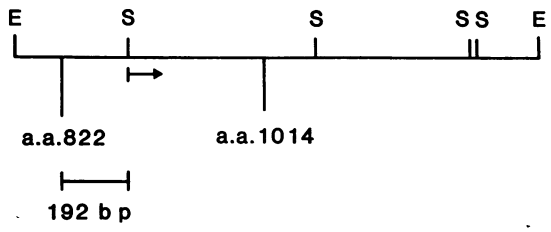


*Figure 5.* Partial restriction map of cDNA clones. Shown are the restriction sites for Sau3AI(S) and EcoRI (E), and the approximate locations of the codons corresponding to the $NH_2$- and COOH-terminal amino acid residues of α1-CB6. The arrow indicates the direction of sequencing from the Sau3AI site.

*Polyacrylamide gel electrophoresis.* To prepare a sample for gel electrophoresis, it was rapidly immersed in boiling water for 3 min with the concomitant addition of a 0.2 vol of 5× electrophoresis sample buffer that consisted of 10% SDS, 50% glycerol, and 0.012% bromophenol blue in 0.625 M Tris/HCl buffer (pH 6.8). Samples were applied to SDS-gels either with or without prior dialysis against 1× sample buffer. Electrophoresis was performed using the discontinuous system of Laemmli (35, 36).

Two-dimensional mapping of cyanogen bromide (CNBr) peptides was performed with the method of Barsh et al. (37) by first digesting cell layer proteins with pepsin and separating the proteins by electrophoresis in SDS on a 5% polyacrylamide gel. Individual lanes from the gel were digested with CNBr, and the samples were again separated by electrophoresis on a 12.5% polyacrylamide gel. For the second dimension, an 8% polyacrylamide stacking gel was employed. Samples were either reduced or not reduced with 5% 2-mercaptoethanol before electrophoresis in the second dimension.

*Synthesis and screening of a cDNA library.* About 5 μg of poly(A)-enriched mRNA was isolated from three 175-cm$^2$ flasks of the proband's fibroblasts and used to synthesize cDNA (38). To increase the yield of cDNAs containing proα1(I) sequences, a 21-bp oligonucleotide complementary to the 3′ region of the proα1(I) mRNA was used as a primer for the first strand synthesis. The oligonucleotide primer was complementary to the last 14 nucleotides coding for the C-propeptide plus seven nucleotides of the 3′ untranslated region (39). EcoRI linkers were attached to the double-stranded cDNA, ligated to λ gt10 arms (Promega Biotec, Madison, WI), and packaged in vitro using Packagene extract (Promega Biotec). The bacteriophage library generated had about 3 × 10$^6$ recombinant clones. The library was screened with the large EcoRI fragment of Hf-677, a cloned cDNA of about 1.7 kb

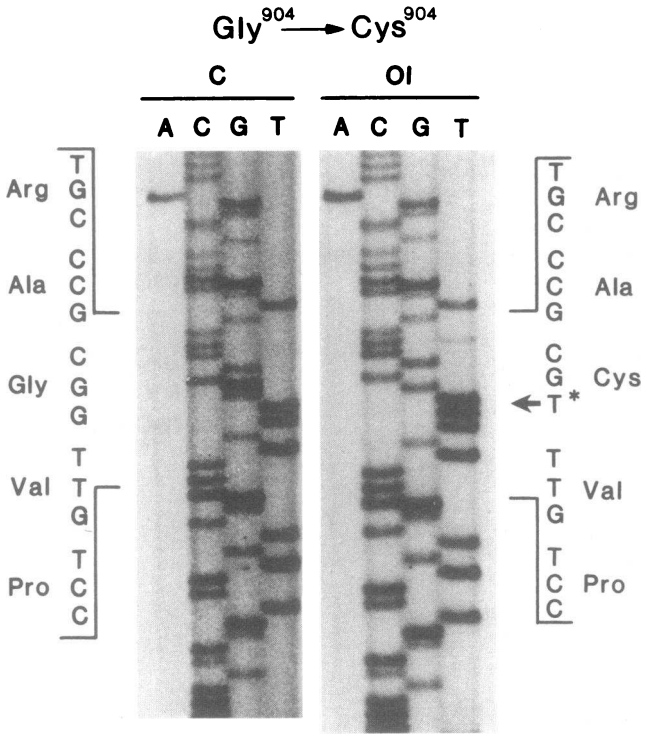


*Figure 6.* DNA sequence of normal (*C*) and mutant (*OI*) cDNA clones. cDNA clones were sequenced from the Sau3AI site (see Fig. 5). The *arrow* points to the thymidine substituting for the normal guanosine.

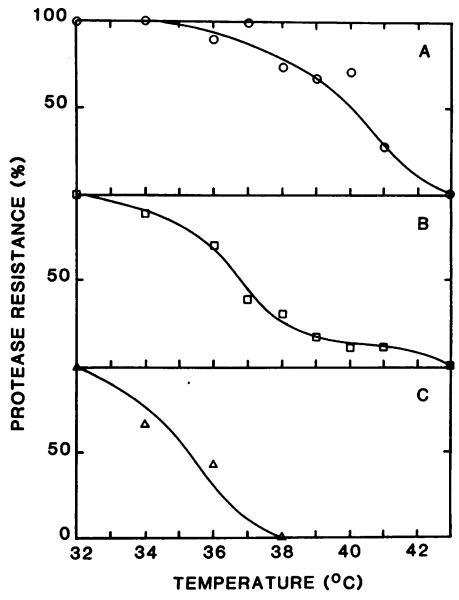


*Figure 7.* Thermostability to brief proteinase digestion of procollagens from control and OI fibroblasts. Radioactively labeled cell homogenates were treated with pepsin and then digested with trypsin and chymotrypsin at various temperatures as described in Methods. (*A*) Melting curve of normal collagens, showing an apparent $T_m$ of about 40°C. (*B*) Melting curve of type I procollagen from proband that did not generate disulfide-linked dimers. The biphasic nature of the curve indicates a mixed population of normal and abnormal molecules, the abnormal ones having an apparent $T_m$ of about 37°C. (*C*) melting curve of OI molecules that generated disulfide-linked dimers showing an apparent $T_m$ of about 35°C.

coding for the 3′ half of the human pro$\alpha$1(I) chain (39). The probe was labeled by nick translation to a specific activity of $10^8$ cpm/$\mu$g and the library was screened by plaque hybridization (40).

Fourteen positive clones were plaque purified and digested with EcoRI. The EcoRI inserts were subcloned into the filamentous bacteriophage M13 mp19. Inserts were digested with Sau3AI, and the 530-bp fragments from the COOH terminus of the $\alpha$-chain domain were subcloned into M13 mp18. The subclones were sequenced with both reverse transcriptase and the Klenow fragment of DNA polymerase I using the dideoxynucleotide method (41) with reagents supplied by two different commercial sources (Promega Biotec and New England Biolabs, Beverly, MA).

## Results

*Identification of a cysteine residue in the $\alpha$-chain domain of the pro$\alpha$1(I) chain.* Control fibroblasts and fibroblasts from the proband were incubated with [$^3$H]proline for 4 h and the cell layer proteins were examined by electrophoresis on a 6% polyacrylamide gel in SDS. As indicated in Fig. 1, most of the pro$\alpha$1(I) and pro$\alpha$2(I) chains of type I procollagen synthesized by the proband's fibroblasts migrated more slowly than the same chains from control fibroblasts. Most of the $\alpha$ chains obtained after pepsin digestion migrated more slowly than $\alpha$1(I) and $\alpha$2(I) chains from control fibroblasts (Fig. 2). The difference in migration was abolished when the proband's fibroblasts and the control fibroblasts were incubated in the presence of 0.3 mM $\alpha,\alpha'$-dipyridyl to inhibit prolyl and lysyl hydroxylases (Fig. 1). Therefore the slower migration of the pro$\alpha$ chains of the proband's fibroblasts is probably explained by posttranslational overmodification of these chains. As indicated in Fig. 2, $\alpha$1 chains of type V collagen (42, 43) comigrated with control $\alpha$1(V) chains. Therefore, the overmodification of the $\alpha$ chains of type I collagen is not due to a generalized abnormality in posttranslational reactions.

In further experiments, fibroblasts from the proband and control were incubated with [$^3$H]proline, and the proteins from the cell layer were first digested with pepsin and then partially digested with vertebrate collagenase before analysis by electrophoresis. As indicated in Fig. 3 (*left panel*), both the collagenase A fragments and collagenase B fragments from the proband's type I collagen migrated more slowly than the same fragments from the control. As expected, the difference in migration was abolished if the control and proband's fibroblasts were incubated with 0.3 mM $\alpha,\alpha'$-dipyridyl (Fig. 3, *right panel*).

Further examination of the same gel (Fig. 3) indicated the presence of a band migrating more slowly than $\alpha$ chains but more rapidly than $\gamma$ chains of type III collagen (not shown). The slowly migrating band was not seen when the samples were reduced prior to electrophoresis. Therefore, the data were consistent with the hypothesis that the slowly migrating band consisted of disulfide-linked dimers of $\alpha$1(I) chains. Densitometry of fluorograms indicated that the putative disulfide-linked dimers accounted for less than 10% of the total $\alpha$1(I) chains.

To define the structure of the putative disulfide-linked $\alpha$1(I) chains, cell layer proteins were first digested with pepsin and the proteins were separated by polyacrylamide gel electrophoresis in SDS in one dimension. The gel lane was incubated with CNBr, and then analyzed by electrophoresis in a second dimension with and without prior reduction. As indicated in Fig. 4 (*right panel*), the reduced sample of putative disulfide-

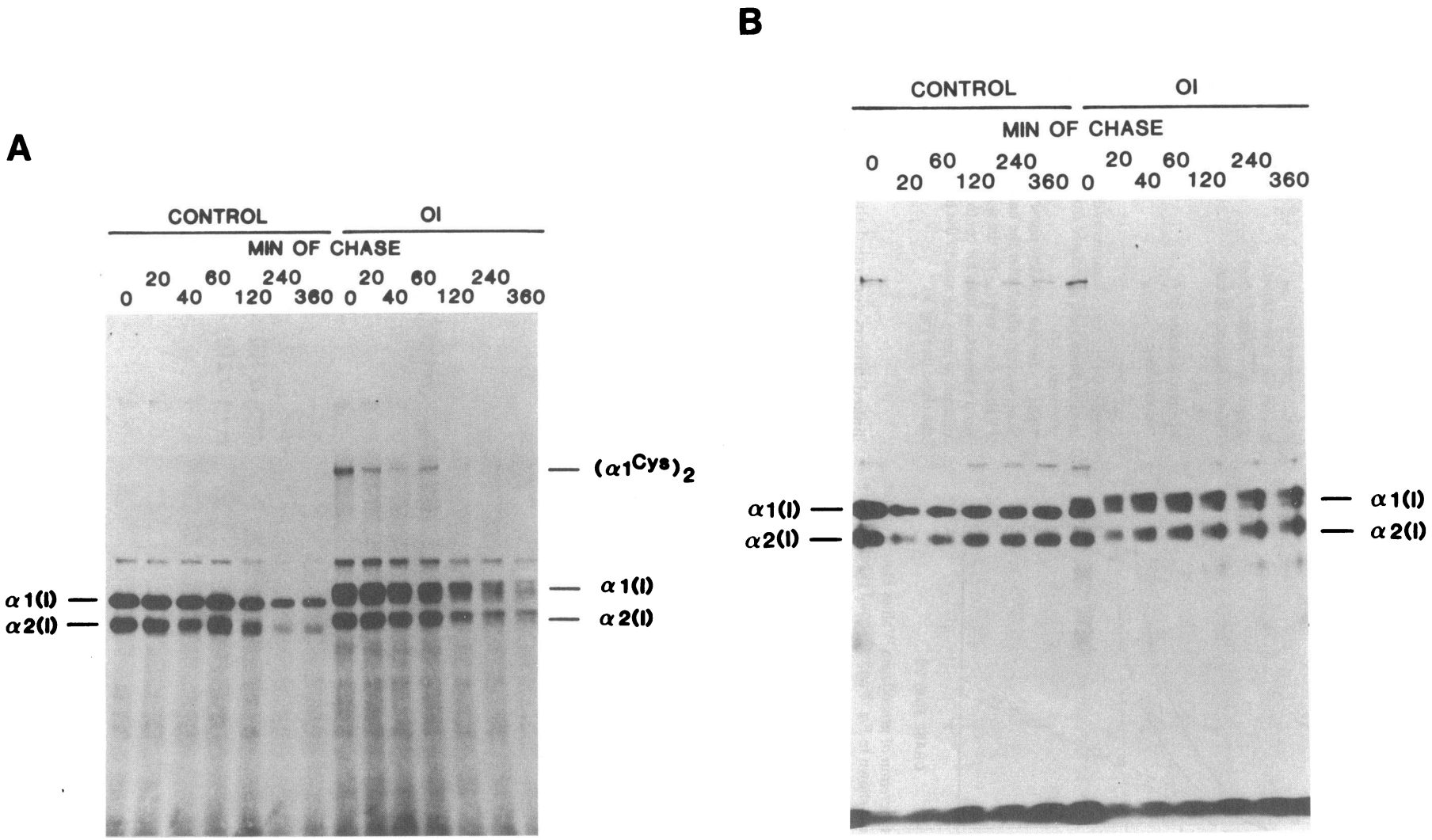


*Figure 8.* Kinetics of secretion of procollagen. Control and OI fibroblasts were labeled for 3 or 4 h with 50 μCi/ml of [$^3$H]proline and then the label was chased (see Methods). Cell homogenates were digested with trypsin-chymotrypsin at room temperature for 2 min and equal aliquots were electrophoresed on a 6% SDS-polyacrylamide gel. (*A*) Samples from cell layers. (*B*) Samples from media.

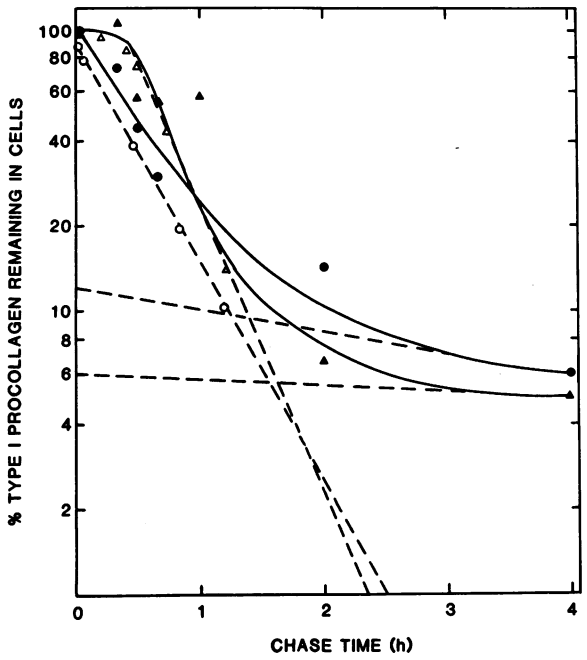


*Figure 9.* Kinetics of secretion of procollagen. Values plotted are the mean from experiment shown in Fig. 8 and a second experiment. (●) control fibroblasts; (▲) proband's fibroblasts; (○) values obtained by exponential peeling of curve for control fibroblasts; (△) values obtained by exponential peeling of curve for proband's fibroblasts. The curve for the proband's fibroblasts does not intersect the abscissa because of the apparent lag period in secretion (see text).

*Table I. Kinetics of Procollagen Secretion by Control and OI Fibroblasts*

| | Control | OI* |
|---|---|---|
| $k_1$ | $3 \times 10^{-2}$ min$^{-1}$ | $3.9 \times 10^{-2}$ min$^{-1}$ |
| $k_2$‡ | $2.9 \times 10^{-3}$ min$^{-1}$ | $0.76 \times 10^{-3}$ min$^{-1}$ |
| $t_{1/2}$ for fast phase (*min*) | 23 | 18 |
| $t_{1/2}$ for slow phase‡ (*min*) | 239 | 912 |
| Fraction secreted in fast phase | 88% | 94% |
| Fraction secreted in slow phase | 12% | 6% |

Values for the rate constants and the fraction of procollagen secreted in each phase were calculated from the data in Fig. 9 as described previously (34).

* The $t_{1/2}$ for fast and slow phase of secretion by the proband's fibroblasts was calculated after the half hour lag period.

‡ Values for $k_2$ and $t_{1/2}$ for the slow phase were different for the proband's fibroblasts than for control fibroblasts, but these values were difficult to assay because of the rapid secretion between 1 and 2 h of the chase (see Fig. 9).

linked dimers generated the same CNBr peptides as α1(I) chains. Therefore the data established that the slowly migrating band consisted of α1(I) chains. However, when the electrophoresis was carried out in the second dimension without prior reduction of the samples (*left panel*), the disulfide-linked

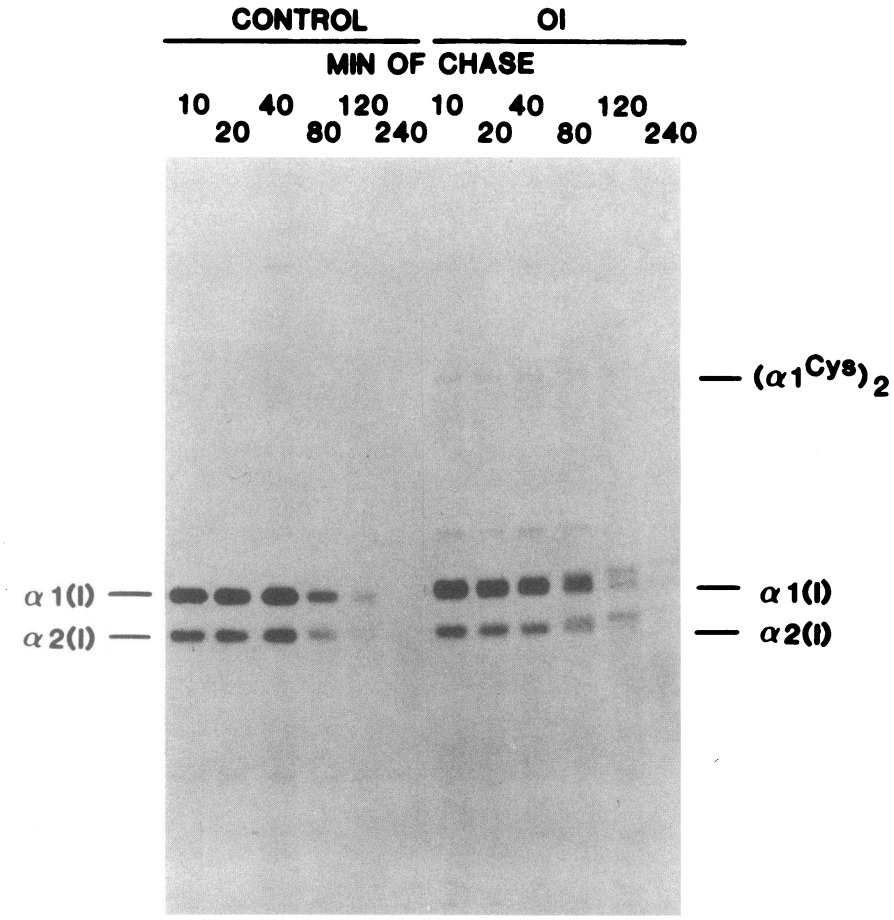


*Figure 10.* Pulse-chase labeling of control (*C*) and proband's fibroblasts (*OI*). Fibroblasts were labeled for 1 h and chased for up to 4 h with unlabeled medium. The samples were digested with trypsin-chymotrypsin before electrophoresis. After 40 min of chase, the intracellular procollagens gradually become overmodified in the OI fibroblasts. A doublet of overmodified and grossly overmodified α1(I) chains is seen at 120 and 240 min.

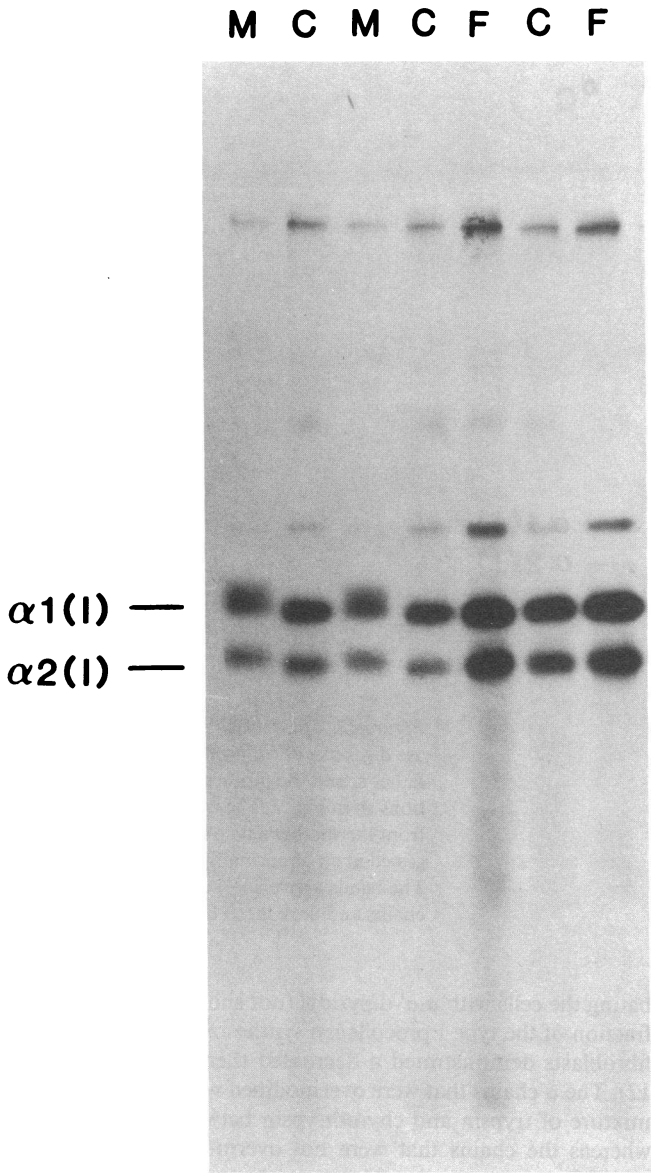


*Figure 11.* SDS-polyacrylamide gel electrophoresis of proteins from control and the parent's fibroblasts. Cell layer homogenates were digested with a mixture of trypsin and chymotrypsin at 20°C and the proteins were electrophoresed on a 5% polyacrylamide gel. *M*, mother's fibroblasts; *C*, control fibroblasts; and *F*, father's fibroblasts. A fraction of the mother's α chains migrated more slowly than the control or the father's α chains.

dimers did not generate a peptide corresponding to α1-CB6, the CNBr fragment containing amino acid residues 823–1014 of the α1(I) chain.

*Cloning and sequencing of the region of the cDNA containing the mutation.* A cDNA library was prepared with mRNA from the proband's fibroblasts, and clones containing coding sequences for the proα1(I) chain were identified. Regions of the cDNAs coding for α1-CB6 were subcloned and sequenced. Preliminary restriction mapping of the EcoRI cDNA inserts (Fig. 5) demonstrated that about half the clones lacked a HaeII site. The missing HaeII site overlapped the codon for the glycine in amino acid position 904 of the α1(I) chain (39). The HaeII site was located between two Sau3AI sites (Fig. 5). Therefore, subclones of the EcoRI inserts were prepared by digesting the inserts with Sau3AI, and the 530-bp Sau3AI fragments were subcloned into M13. Two subclones were repeatedly sequenced with both reverse transcriptase and the Klenow fragment of DNA polymerase I (Fig. 6). One of the subclones was from a clone of the proband's cDNA that contained the expected HaeII site. The other was a subclone from a clone of the proband's cDNA that did not contain the HaeII site. At the same time, sequencing was performed on analogous Sau3AI fragment from Hf-677, the cDNA for a normal proα1(I) chain previously analyzed (39). The results established one critical difference among the subclones. As indicated in Fig. 6, there was a substitution of T for G in the codon for the glycine in amino acid position 904 of the α1(I) chain in the proband's cDNA clone that lacked the HaeII site. The substitution of T for G converted the glycine codon to a codon for cysteine.

In addition, the results defined three errors in the previous nucleotide sequence for this same region. In all three subclones, including the subclone from Hf-677, the codon for amino acid 903 was found to be -GTT- and not -GCT-. Therefore the amino acid in position 903 is valine and not alanine as previously reported (39). The two additional errors were that in all three clones the proline codons for amino acid positions 899 and 902 were -CCT- and not -CCC- as previously reported (39).

*Thermostability of the proband's type I collagen.* To demonstrate that the mutation changed the functional properties of the type I collagen, the helical stability of the collagen synthesized by the proband's fibroblasts was examined by controlled digestion with a mixture of trypsin and chymotrypsin (31, 32). As reported previously (36), the type I collagen from the proband's fibroblasts had a decreased thermostability (Fig. 7). As indicated in Fig. 7 *B*, the type I collagen that did not generate disulfide-linked dimers had a biphasic melting curve. Most of the protein had an apparent midpoint temperature for the helix-to-coil transition ($T_m$) of 37°C or about 3–4° lower than the apparent $T_m$ of type I collagen from the control fibroblasts (Fig. 7 *A*). However, a fraction of the protein had a normal thermal stability. The fraction of the collagen that generated disulfide-linked α1(I) chains had an apparent $T_m$ of about 35°C, or about 5° lower than the control collagen (Fig. 7 *C*). Comparison of bands in the same lanes in fluorograms exposed for varying times (not shown) confirmed the impression that the disulfide-linked species had a lower $T_m$ than the over-modified α1(I) chains without disulfide links.

*Kinetics of secretion of procollagen.* To examine the kinetics of secretion of type I procollagen, control and OI fibroblasts were each labeled for 3 or 4 h with [$^3$H]proline and the label was chased (34).

As indicated in Figs. 8 and 9, procollagen secretion by the proband's fibroblasts followed an unusual kinetic pattern. At the end of the pulse period, there was less overmodified than normal procollagen in the medium (Fig. 8 *B*). During the chase period, progressively more of the procollagen was overmodified. Densitometric analysis of the fluorograms indicated that there was a lag period of about 30 min followed by relatively rapid secretion of the protein (Fig. 9). If corrected for the lag period, the rate constant for the first phase of secretion, $k_1$, was about the same as for control fibroblasts (Fig. 9 and Table I). The second rate constant, $k_2$, was about one-quarter the value for the control fibroblasts, but because of the rapid se-

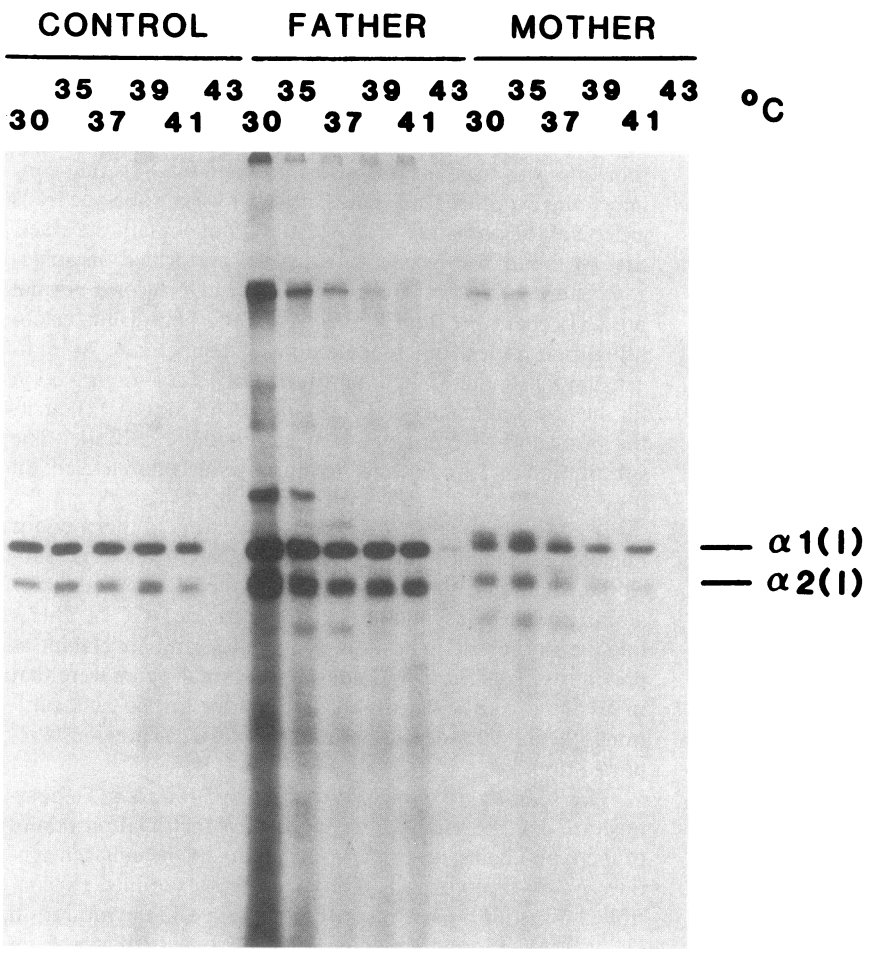


*Figure 12.* Thermostability to brief proteinase digestion of collagens from control, the father's, and the mother's fibroblasts. Conditions as in Fig. 7. The overmodified α chains from the mother's type I collagen were digested at an abnormally low temperature. The bands above α1(I) chains are α1(V) chains and unreduced α1(III) chains.

cretion between about 1 and 2 h of chase, the value was difficult to estimate accurately. The procollagen that gave rise to disulfide-linked dimers was not consistently detected in the medium.

In order to examine in more detail the behavior of abnormal molecules during the early events of synthesis and secretion, the experiment was repeated with a pulse-labeling period of only 1 h. As indicated in Fig. 10, during the apparent lag phase in secretion, there was progressive overmodification of the intracellular α chains. After a chase of 120 or 240 min, the band of α1(I) chains appeared as a doublet. Both components of the doublet migrated more slowly than α1(I) chains from control fibroblasts. Hence, the apparent lag was probably explained by time required for procollagen protomers containing one or two abnormal proα1(I) chains to become overmodified and to fold into a triple-helical conformation.

*The mother's fibroblasts synthesize an overmodified and unstable species of type I procollagen.* In further experiments, the type I procollagen synthesized by the parents' fibroblasts was examined. The father's fibroblasts synthesized an apparently normal type I procollagen in that there was no evidence of posttranslational overmodification of α chains (Fig. 11) and no evidence of a disulfide-linked dimer (Figs. 11 and 12). Also, the thermal stability of the α chains was normal (Fig. 12). In contrast, a fraction of the α chains synthesized by the mother's fibroblasts was overmodified. As indicated in Fig. 11, about one-quarter of the α1(I) and α2(I) chains migrated more slowly, but the difference in migration was abolished by incubating the cells with α,α′-dipyridyl (not shown). In addition, a fraction of the type I procollagen synthesized by the mother's fibroblasts demonstrated a decreased thermal stability (Fig. 12). The α chains that were overmodified were digested by the mixture of trypsin and chymotrypsin between 37 and 39°C, whereas the chains that were not overmodified were stable to 41°C.

## Discussion

The mutation described here is the fourth reported example of a type I procollagen with an amino acid replacement for glycine in the α1(I) chain with cysteine or arginine (22–25, 44). The four mutations span amino acids 391–988 of the α1(I) chain. Because all four probands died *in utero* or shortly after birth, the results raised the possibility that any mutation that substitutes a bulkier amino acid for glycine in the α1(I) chain sufficiently disrupts the conformation of the protein to produce a lethal phenotype.

The glycine substitution described here had several features similar to those found with the three previously reported glycine substitutions in the α1(I) chain (22–25, 44), but at least one critical feature that differed. As with the two previously reported mutations in which thermal stability was tested (23, 24), the thermal stability of the mutated type I procollagen was lowered, and the fraction of the protein containing two disulfide-linked α1(I) chains had an even lower thermal stability than the fraction with only one glycine-substituted α1(I) chain.

Also, there was a decrease in the rate of secretion of the procollagen. The decrease in rate of secretion appeared to consist of a lag period in the pulse-chase experiments followed by secretion with approximately normal apparent constants. The glycine to cysteine mutation was not found in type I procollagen from either parent. Therefore, the cysteine mutation was a sporadic one. Examination of the type I procollagen synthesized by the mother's fibroblasts indicated that a fraction of the pro$\alpha$ chains were overmodified and had a lower thermal stability. Therefore, the results raised the possibility that the proband inherited a mutated allele for either pro$\alpha$1(I) or pro$\alpha$2(I) chains from her mother. Accordingly, the cysteine mutation may not in itself have produced the lethal phenotype. A similar situation was encountered in studies on the lethal variant of OI with a substitution of cysteine for glycine 988 of the $\alpha$1(I) chain because the contribution of a mutation producing the Marfan syndrome in the mother could not be defined (24, 25). Unfortunately, it will also be difficult to generate definitive data about the mutation in type I procollagen genes in the mother studied here. Preliminary experiments with vertebrate collagenase (36) did not indicate whether the mother's mutation changes the thermal stability of the A or the B fragments (not shown). Therefore, it will be necessary to sequence extensively cDNAs or genomic DNAs from her fibroblasts to identify the mutation.

The observations made with the mother's fibroblasts raise several questions about the causal relationship between mutations in the genes for type I procollagen and heritable disorders of connective tissue such as OI. She is the first asymptomatic individual whose fibroblasts were found to synthesize a type I procollagen that is both overmodified and has a lowered thermal stability. She may well have a minor deficiency in the structure of bone and other tissues that are rich in type I collagen. Therefore she may well be predisposed to conditions such as osteoporosis in the future (1, 45). However, she was asymptomatic at age 35. She had slightly blue sclerae and was somewhat small, but physical examination as well as bone densitometry did not reveal any marked abnormalities. Therefore, the observations on the mother indicate that a mutation causing synthesis of a type I procollagen with a lowered thermal stability does not necessarily produce a heritable disorder of connective tissue.

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*Note added in proof.* Recently, we found that passage 7 fibroblasts from the proband's mother synthesize a small amount of disulfide-linked $\alpha$1(I) chains and that the mother also has the substitution of a cysteine codon for glycine 904 in some of her mRNA for pro$\alpha$1(I) chains. Therefore, the mother may be a mosaic for the mutation and her fibroblasts with the mutation are lost in the later passage cultures (about 10–14) used for the experiments described here.

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