Previously we showed that fibrils generated from collagen and pNcollagen-** chains from fibroblasts of an individual with Ehlers-Danlos syndrome (EDS) type VIIB were hieroglyphic in cross-section and all N-propeptides were located at the fibril surface. Hieroglyphs were resolved near-cylindrical fibrils (that were similar in appearance to the fibrils seen in the tissues of individuals with EDS type VIIB) by treatment with N-proteinase which cleaved the pNal(1) chains but not the pNa2(1)-** chains (Watson, R. B., Wallis, G. A., Holmes, D. F., Viljoen, D., Byers, P. H., and Kadler, K. E. (1992) J. Biol. Chem. 267, 8093–9100).

Here, quantitative scanning transmission electron microscopy (STEM) showed that N-propeptides in hieroglyphs were in a “bent-back” conformation and thus located exclusively in the overlap zone of the fibril D-period (D = 67 nm). In contrast, STEM of fibrils from the dermis of an individual with EDS type VIIB showed that partially cleaved N-propeptides (in which cleaved pNal(1) remained in noncovalent association with pNa2(1)-** chains) were distributed equally between the gap and overlap zones of the fibrils. Comparison of experimental data with theoretical mass distributions of the fibril based on amino acid sequence data gave a consistent value of 33 nm for the total axial extent for the N-propeptides in hieroglyphic and tissue fibrils irrespective of the location of N-propeptides to the gap or overlap zone. These data exclude the possibility that N-propeptides adopt a random configuration, but rather, that they locate to specific sites in the gap and overlap zones. The results demonstrated that cleavage of pNal(1) chains in vivo releases the N-propeptides from the constraints of the bent-back conformation. Co-distribution of partially cleaved N-propeptides between gap and overlap zones allows a higher surface packing density of N-propeptides and explains how circularity of large diameter fibrils can be achieved despite the retention of N-propeptides in tissues of individuals with EDS type VIIB.

EDS, a heterogeneous group of heritable disorders characterized by hypermobility of joints and abnormalities of skin, is classified into 10 types on the basis of clinical and biochemical findings (Beighton et al., 1988). EDS type VII is inherited in an autosomal dominant fashion and is distinct from other forms of EDS by virtue of marked joint hypermobility, multiple joint dislocations, and congenital hip dislocations that are usually bilateral. The biochemical basis of the disorder is a failure to process completely the N-propeptides of type I procollagen (for reviews see Byers, 1989; Steinmann et al., 1993) because of mutations that result in skipping of exon 6 during processing of pre-mRNA for either the proal(1) or proal(2) chain of type I procollagen.

Studies of individuals with EDS type VII have shown that the collagen fibrils in skin, bone, and facia of these people are near-circular in cross-section. Also, collagen extracted from skin and pepsin-treated procollagen secreted from fibroblasts in culture contained the mutant pNa2(1)-** chain and normal sized al(1) chains (Eyre et al., 1985; Wirtz et al., 1987). The fact that pNa1(1) chains were not observed indicated that the al(1) N-propeptides had not been proteolytically cleaved from the molecule. However, subsequent morphological, chemical, and immunochemical studies showed that, despite proteolytic cleavage, the excised al(1) N-propeptides remained in noncovalent association with mutant pNa2(1)-** chains in vivo and in vitro (Wirtz et al., 1990). Of particular relevance to our studies, the cleavage of pNa1(1) chains does not alter the mass of the partially cleaved N-propeptides in molecules of pNcollagen-** (McPherson et al., 1987) from the 18 daltons introduced by proteolysis of each proal(1) chain.

Previously we showed that an individual with EDS type VIIB had a G to A transition at the 5' donor splice site of exon 6 in one allele for the COL1A2 gene (Watson et al., 1992). As type I procollagen comprises two proal(1) and one proal(2) chains in vivo, the 5' donor splice site of exon 6 of this allele is in the COL1A2 gene.
proa2(I) chains, half of the individual's type I procollagen was expected to be normal, and half was expected to contain abnormal proa2(I) chains that lacked the sequences encoded by exon 6 containing the site for cleavage by N-proteinase. Subsequent analysis showed that half of the type I procollagen secreted by the individual's dermal fibroblasts in culture was cleaved normally by N-proteinase, and half was resistant to the enzyme. Cleavage of the resultant mixture of pCollagen and uncleaved procollagen by C-proteinase in vitro generated fibrils that were 1:1 co-polymers of collagen and pNcollagen-α2(I) and which were highly irregular in cross section and similar to the hieroglyphic fibrils seen in the skin of dermatosparaxic animals (Lemaers et al., 1971; Counts et al., 1980; Holbrook et al., 1980; Fjellstad and Helle, 1974) including humans (Nugents et al., 1992; Smith et al., 1992).

The hieroglyphic appearance of the fibrils and the persistence of intact pNααααα(I) chains was in marked contrast to what has been observed in tissues of individuals with EDS type VIIIB. In further analysis, treatment of the hieroglyphs with N-proteinase resulted in cleavage of pNααααα(I) chains but not pNααααααααα(I) chains in abnormal molecules and the hieroglyphs spontaneously resolved to fibrils that were near-circular in cross-section. The resolved fibrils resembled the near-cylindrical collagen fibrils seen in tissues of individuals with EDS type VIIIB both in terms of morphology and molecular composition. These observations suggest that the gross differences in morphology between hieroglyphic and near-cylindrical fibrils are a result of conformational changes in the N-propeptides resulting from cleavage of pNααααα(I) chains.

Here we have used quantitative STEM to examine the conformation of the N-propeptides in fibrils from the skin of an individual with EDS type VIIIB who had a mutation in the COL1A2 gene that caused skipping of exon 6 during processing of mRNA (Wirtz et al., 1987, 1990) and in hieroglyphic fibrils generated in vitro from collagen and pNcollagen-α2(I). We show that uncleaved N-propeptides locate exclusively to the overlap zone of the D-period in hieroglyphics, whereas in the EDS type VII skin fibrils the N-propeptides equally co-distribute between the gap and overlap zones. Implications of the distribution of N-propeptides within the fibril D-period are discussed with relevance to fibril morphology.

**MATERIALS AND METHODS**

**Source of Materials**

Radiochemicals were from ICN Radiochemicals; sodium ascorbate was from Sigma; Dulbecco's modified Eagle's medium was from ICN-Flow; DEAE-cellulose was from Whatman; YM100 ultrafiltration membranes were from Amicon; Sephacryl S-300 resin was from Pharmacia-LKB Ltd.; fertile hen eggs were from Northern Biological Supplies (St. Annes, United Kingdom); spectroscopically pure carbon (rods) and copper grids were from Agar Scientific (Stanstead, United Kingdom); water used in the preparation and analyses of the proteins was from a commercial water purification system that comprised tap water feeding into a Millipore R06. Electron Microscopy of Negatively Stained Fibrils

Fibril samples were adsorbed to carbon-filmed grids for 15 s, washed with water and dehydrated to 1% phosphotungstic acid (pH 7.4) for 10 s. The grids were examined in a JEOL 1200 EX transmission electron microscope operated at 120 kV. Images were recorded at ×50,000 magnification on Ilford E.M. film.

**Preparation of Collagen**

1C-Labeled type I procollagen was purified from the culture medium of dermal fibroblasts from an individual with EDS type VIIIB (Watson et al., 1992) using the methods described previously (Kadler et al., 1987). In brief, fibroblasts were grown to confluency and incubated in Dulbecco's modified Eagle's medium supplemented with 1 μCi/ml of a mixture of uniformly labeled C-1 amino acids, 25 μg/ml ascorbic acid, and no serum. Proteins in the culture medium were then precipitated by ammonium sulfate, and the precipitates were dialyzed against a colormetric hydroxyproline procedure (Woessner, 1961), assuming 10.1% hydroxyproline by weight procollagen (Fjellstad-Nagy et al., 1981). The procollagens had a specific radioactivity of 1500 cpm/μg.

**Preparation of Hieroglyphic Fibrils**

Purified 1C-labeled type I procollagen (from EDS type VIIIB cells in culture) was incubated with N-proteinase and the resultant mixture pCollagen/uncleaved procollagen, and C-proteinase, were dialyzed separately against 2 x 600 volumes of fibril formation buffer consisting of 20 mM NaHCO3, 117 mM NaCl, 3.4 mM KCl, 1.8 mM CaCl2, 0.81 mM MgSO4, 1.03 mM NaH2PO4 and 0.01% NaN3 (pH 7.4 at 20°C). The substrate (100 μg/ml) and C-proteinase (50 units/ml) were mixed in a 1.5 ml microcentrifuge tube, and 3-μl aliquots of the fibril formation mixture were suspended from a small piece of polystyrene-covered wire glued into the cap of a glass Bijou tube. This “hanging-drop” method maximized recovery of fibrils on EM grids. Five ml of fibril formation buffer was added to the tube to prevent changes in pH and volume of the sample. To initiate fibril formation the tube was submerged in a water bath at 37°C for 24 h.

**Preparation and Analysis of Tissue Fibrils**

Samples of dermis of an individual with EDS type VIIIB were obtained at surgery and stored frozen. The clinical details and molecular characterization of the defect have been described elsewhere (Steinmann et al., 1985; Wirtz et al., 1987, 1990; Steinmann et al., 1992). The mutation was a T to C transversion at the 5' donor splice site of exon 6 in one of the patient's COL1A2 alleles that resulted in skipping of exon 6 sequences during processing of pre-mRNA for the proa2(I) chain. Several 1-mm² pieces of dermis from the individual with EDS type VIIIB and a 2-year-old heifer were separately frozen in liquid nitrogen and fragmented in a microtome bomb precooled by immersion in liquid nitrogen. The powder was suspended in phosphate-buffered saline and homogenized for 2 x 1 s. The homogenate was centrifuged at 15,000 x g for 6 min and the pellet resuspended in 100 μl of phosphate-buffered saline. Large fragments of tissue were removed by centrifugation of the sample at 15,000 x g for 15 s. The resultant fibril suspension was recovered into a fresh microcentrifuge tube. For analysis of the chain composition of the fibrils, samples were prepared for SDS-PAGE in Laemmli buffer (Laemmli, 1970) and separated on 7% polyacrylamide gels in the presence of SDS. Proteins were visualized by Coomassie Blue and destained in 30% methanol and 10% acetic acid. For electron microscopy, the fibril suspension was treated with formaldehyde (1% final concentration) for 30 min at room temperature.

**Electron Microscopy**

Electron Microscopy of Negatively Stained Fibrils

Fibril samples were adsorbed to carbon-filmed grids for 15 s, washed with water and dehydrated to 1% phosphotungstic acid (pH 7.4) for 10 s. The grids were examined in a JEOL 1200 EX transmission electron microscope operated at 120 kV. Images were recorded at ×50,000 magnification on Ilford E.M. film.

**Scanning Transmission Electron Microscopy**

*Use of a Conventional Instrument in the STEM Mode—Fibrils* were adsorbed to 600 mesh carbon-filmed grids, washed with six drops of ultrapure water, and air-dried. The unstained fibrils were examined by STEM. The basic instrument was a JEOL 200CX transmission electron microscope equipped with a JEOL ASID10 scanning unit and a standard bright field and an annular dark-field detector. The instrument was interfaced with a microcomputer system (Holmes et al., 1991a) that permitted digital control of the scan and digitization.

**Procollagen N- and C-proteinases**

The C-proteinase was purified from the culture medium of the leg tendons of 250 dozen 17-day chick embryos as described previously (Hojima et al., 1985). The preparation had 400 units/ml of activity where 1 unit is the amount required to cleave 1 μg of type I procollagen at 34°C in a reaction system containing procollagen at a concentration of 10 μg/ml. The N-proteinase was purified from extracts of the used tendons as described previously (Hojima et al., 1989). The preparation had 800 units/ml of activity.
of the signal from the dark-field detector. The STEM was operated at 120 kV with the standard lens settings. The collection angle of the annular dark-field detector ranged from 25 × 10−3 to 75 × 10−3 radians. The signal from the dark-field detector was linearly dependent on carbon film mass thickness up to approximately 50 kDa·nm−2. Images of Fibrils—Mass mapping procedures were similar to those developed for a dedicated STEM instrument with a field-emission gun (Engel, 1978, 1982; Engel et al., 1981; Engel and Reichelt, 1984). Digital images were acquired using an approximately 3-nm spot size and exposing the specimen to a low electron dose (<105 e/Å2). A diffraction grating replica (2160 lines/mm) was used for magnification calibration and was estimated to be accurate to better than 2%. At the instrumental magnification setting of ×80,000 used here, the pixel size was 2.5 nm. Specimens were at room temperature during electron microscopy.

**Determination of the Molecular Polarity of the Fibrils**

Grids used for obtaining STEM images were post-stained with 1% aqueous uranyl acetate (pH 4.2) for 20 s. Grids were washed with three drops of ultrapure water and air-dried. When re-examined by conventional transmission electron microscopy a characteristic staining pattern was observed in those regions of fibrils that had received an electron dose of not more than 1 electron nm−2. Inspection of the positive staining pattern adjacent to the region of the fibril used to collect STEM data allowed fibrils to be assigned a molecular polarity. No differences were observed in the positive staining patterns of the control, hieroglyphic, and EDS type VIIb fibrils.

**Determination of the Axial Mass Distribution (AMD) of the D-Period**

Digital images were analyzed using procedures written within the SEMPER V Image Analysis program (Synoptics Ltd., Cambridge, United Kingdom) and run on a 33-MHz 486-based microcomputer (Tiko PXC433). Digital densitometric scans were made along the axis of fibrils. Typically, scans were made covering 10 D-periods in length and 20–50 nm in width depending on the width of the fibril. Multiple scans were made in register across the fibril. This was particularly important for analysis of hieroglyphs where the mass contrast of the gap/overlap varies across the fibril arising from the surface location of N-propeptides and collapse of the hieroglyphs during drying on the grid.

To generate an average axial mass distribution for the D-period of each fibril sample, three stages of averaging were employed. First, an internal average for the D-period was prepared from each digital densitometric scan covering 10 periods. Second, internal averages across the fibril were combined to yield a fibril average. Finally, fibril averages were oriented in the N-to-C direction and combined to generate a sample average of the AMD of the fibril D-period. More than 100 measured D-periods from a number of fibrils contributed to the final averages of each sample. The axial alignment step required to generate fibril and sample averages was achieved by maximizing the cross-correlation coefficient.

The mass distribution of N-propeptide mass along the fibril axis, AMDs of control fibrils were subtracted from AMDs generated for hieroglyphs and EDS type VIIb fibrils. However, to do this the three AMDs had to be scaled according to the average molecular mass of molecules comprising the fibrils. Biochemical data indicated that the hieroglyphs and EDS type VIIb skin fibrils contained approximately 50% pNcollagen. Therefore 307.5 kDa (molecular mass of collagen = 290 kDa, molecular mass of pNcollagen = 325 kDa) was used as the mean molecular mass of a molecules within the hieroglyph and EDS fibrils. AMDs of control fibrils were scaled to produce an average of 100. The mean of the EDS fibril AMDs was then set to 307.5/290 × 100.

**Theoretical AMDs of the D-Period**

A FORTRAN program ("TAMP") was written to generate the theoretical AMD of a D-period using amino acid sequence data. The procedure was analogous to that used to generate the theoretical staining pattern of the type I collagen fibril (Chapman and Hardcastle, 1974).

The amino acid sequence of human type I collagen (Bernard et al., 1983; de Wet et al., 1987; D'Alessio et al., 1988; Kuivanieni et al., 1988; Tromp et al., 1988) was converted to a linear array of numbers known mass and defined axial extent based on the conformational models by Helseth et al. (1979) for the N-telopeptide and Capaldi and Chapman (1982) for the C-telopeptide (see Fig. 5). To generate an average AMD for the fibril D-period, the mass sequences of the five molecules were aligned and mutually staggered by 234 residues determined by Capaldi et al. (1979) and then summed at each residue position along the final averages of each sample. The axial alignment step required to give a mean value of 100 to match the set mean of the experimental data for normal fibrils.

To obtain a best fit between normal and theoretical AMDs a differential contraction was applied to the gap zone. Here, the predicted mass distribution within the gap zone was contracted by 12% in an axial direction. To compensate for the shorter gap zone the AMD was then uniformly extended to its original length. Therefore, in the modified theoretical AMD, the total mass remained unchanged but the average mass per unit length of the gap region increased accordingly.

In analysis of hieroglyphs and EDS type VIIb fibrils, the N-propeptide was considered to comprise three structural domains: a triple helical domain located at its N-end by a globular domain at its C-end by nonhelical extension peptides (NHE) (see Fig. 5B). In all analyses, the α1(I) N-propeptide chains were considered to be associated with pNα2(I)−chains (Wirtz et al., 1990) and in a native triple helical and globular domain substructure. The axial extent of the triple helical domain was calculated from the known residue spacing (h) of 0.286 nm (Meek et al., 1979). The axial extent of the globular region was varied to obtain the best match to experimental data. The theoretical AMDs of hieroglyphic and EDS type VIIb fibrils were scaled to give a mean value of (307.5/290) × 100 to match the set mean of the experimental data for these fibril types.

**RESULTS**

**Analysis of Fibril Composition**—To examine the molecular composition of the hieroglyphic fibrils, fibrils were generated by cleavage of pCcollagen/procollagenα2(I) (100 μg/ml) with C-proteinase (50 units/ml) at 37 °C for 24 h. The fibrils were collected by centrifugation at 15,000 × g for 5 min and analyzed by SDS-PAGE and floouroscopy (Fig. 1). The fibrils contained pNα1(I), α2(I)−chains, α1(I), and α2(I) chains. Quantitation of the amounts of pNα1(I) and pNα2(I)−chains in fibrils by laser densitometry of fluorograms and correction for molecular mass showed that pNcollagen− accounted for 50 ± 5% S.D. (n = 3) of the protein in the fibrils.

To examine the molecular composition of preparations of bovine skin and EDS type VIIb skin fibrils, samples were analyzed by SDS-PAGE and polypeptide chains were detected by Comassie Blue staining of the gel (Fig. 1). Relatively large amounts of skin were needed to obtain sufficient quantities of fibrils for the present study, and adequate amounts of normal human skin were not available. Bovine skin provided a suitable source of type I collagen fibrils in control experiments. In initial experiments, where small amounts of human tissue were available, SDS-PAGE and electron microscopy did not detect any differences between bovine and human skin fibrils. The bovine skin sample contained α1(I) and α2(I) chains and slowly migrating chains that were consistent with the presence of covalently linked dimers of α1(I) and α2(I) chains. Chains of α1(III) collagen were detected. The EDS skin sample contained α1(I), pNα2(I)−chains, and α2(I) chains. A smaller fraction of dimers, compared with the bovine fibrils, was noted. A small amount of α1(III) was present. Quantitation of the pNα2(I)−chains and α2(I) chains by laser densitometry of gels and correction for molecular mass showed pNα2(I)−chains accounted for 53% of the α2(I) plus pNα2(I)−chains. However, because some α2(I) chains were likely to be incorporated in dimers and pNα2(I)−chains were not because of the lack the cross-linking site encoded in exon 6, the ratio...
Hieroglyphic fibrils were generated in a cell-free system by cleavage of a mixture of "C-labeled pCollagen and procollagen"*, collected by brief centrifugation at 15,000 x g, and analyzed by SDS-PAGE and fluorography. Samples of the fibril preparations of normal and EDS VIIB skin fibrils, as used for electron microscopy, were analyzed by SDS-PAGE under reducing conditions and Coomassie Blue staining of the gels.

Mas per Unit Length Measurements of Unstained Fibrils—Quantitative dark-field STEM of normal and EDS type VIIB skin fibrils were used to determine the axial distribution of mass within the fibril D-period for each of the three fibril types. Fig. 3 shows typical dark-field STEM images. Fig. 4 (solid lines) shows average AMDs derived from these and similar images. The AMD of hieroglyphic fibrils (Fig. 4B) shows an enhanced mass contrast when compared with the AMD of normal fibrils (Fig. 4A). The difference between the relative mass/unit length of overlap and gap zones of hieroglyphs (117 minus 87 equals 30, see bold line in Fig. 4B) was approximately double the difference for normal fibrils (107 minus 92 equals 15, see bold line in Fig. 4A). However, there was no difference between the gap/overlap contrast of the EDS skin fibrils and the normal fibrils (compare bold lines in Fig. 4, A and C).

Determination of the Axial Distribution of N-propeptides—The strategy used to determine the axial distribution of N-propeptides in hieroglyphic and EDS skin fibrils was to subtract the AMD of normal fibrils from the AMDs of hieroglyphic and EDS fibrils. However, prior to subtraction, experimental AMDs were precisely aligned with theoretical AMDs derived from amino acid sequence data of human type I collagen and type I pNcollagen as described under "Materials
The best fit between normal and theoretical AMDs was obtained by smoothing the theoretical AMD to a resolution of 15 nm and applying a differential gap contraction of 12% (compare dotted and solid lines in Fig. 4A). The values of 15 nm resolution and 12% gap contraction also provided a good fit between theoretical and experimental curves for the hieroglyphic and EDS skin fibrils. In generating the theoretical curves for hieroglyphic and EDS skin fibrils, we considered the N-propeptide to be comprised of three structural domains extended along the fibril axis (see Fig. 5). The length of the triple helical domain of the N-propeptide was taken to be 13 nm, i.e. 45 × h, where 45 is the number of residues in each chain and h is the residue spacing (h = 0.286 nm). The globular domain of the N-propeptide contains 188 residues (90 in each proc1(I) chain and 8 in the proc2(II) chain) of unknown conformation. Analyses of EDS type VIIB skin fibrils used AMDs in which the a1(I) N-propeptides were considered to be in noncovalent association with pNa2(II) chains, in line with the findings of Wirtz et al. (1990). For simplicity, the 188 residues were considered to conform to a uniform block whose dimensions could be varied for the theoretical analyses. The dotted lines in Fig. 4, B and C, are the theoretical AMDs generated when the globular domain was considered to extend 60 × h (17 nm) and 80 × h (23 nm). In subsequent comparisons of experimental and theoretical data an axial extent of 70 × h for the N-propeptides was found to best fit the experimental data (shown below).

Precise axial registration of the theoretical and experimental AMDs for the hieroglyphs and EDS fibrils was achieved by aligning the NH₂-terminal sides of the overlap regions (as this is unaffected by variations in the axial extension of the N-propeptide). The alignment of the three experimental curves with their theoretical counterparts allowed subtraction of the normal fibril experimental data from the hieroglyphic and EDS fibril experimental data. This yielded the distribution of N-propeptide mass within the D-period for both hieroglyphic and EDS fibril types (Fig. 6, curves 1 and 2, respectively). Since the hieroglyphic and EDS skin fibrils lacked the
FIG. 5. Mass and assigned axial extents for collagen/pNcollagen. A, data for the triple helix and telopeptides of the human type I collagen molecule. The values in brackets are for the triple-helical and N-telopeptide regions of the EDS type VIIB pNcollagen molecule. The reduction in mass values is due to the missing amino acids encoded by exon 6 in the pNa2(I) chain. B, data for the three domains of the N-propeptide of EDS type VIIB pNcollagen. N-T, NH2- terminal telopeptide; C-T, COOH-terminal telopeptide; NHE, nonhelical extension; h equals 0.286 nm.

FIG. 6. Axial mass distribution of the N-propeptide. The AMDs of the N-propeptide were produced by subtraction of the experimental normal AMD from the experimental AMDs of the hieroglyph and EDS fibrils. Curve 1 shows the resulting AMD for the hieroglyphic fibrils. Curve 2 shows the resulting AMD for the EDS type VIIB skin fibrils. The correction curve to compensate for the exon 6 encoded-residues is shown by the dotted line.

sequences encoded by exon 6, the subtraction of the mass distribution of normal from hieroglyph and EDS skin fibrils introduced an error in determining the precise mass distribution of the N-propeptides. The correction curve for these sequences is shown in Fig. 6.

The solid line in Fig. 7B shows the corrected axial mass distribution of the N-propeptides in the EDS skin fibrils. The line shows a near-mirror symmetry about the junction of the gap and overlap zones, implying both an equal distribution of N-propeptide between the gap and overlap regions and a similar structure independent of axial orientation. To estimate the length of the N-propeptide, we compared the distribution of the N-propeptide mass (corrected for lack of exon 6 sequences and smoothed) with a series of theoretically predicted distributions with varying extents (E) of the globular domain of the N-propeptide. The best match was obtained when the globular domain was considered to extend 70 × h, i.e. 20 nm (Fig. 7). (For comparison, a value for E of 60 × h (long dashes) produced a pronounced dip in the curve; a value for E of 80 × h (short dashes) produced a peak in the curve.) In this model, the total axial extent of the N-propeptide was 33 nm and was comprised of 20 nm for the globular domain and 13 nm for the triple helical domain. Noteworthy, this model predicts that opposing N-propeptides (on pNcollagenex molecules separated by 1D on the fibril surface) are in close contact.

The experimentally determined curve for the AMD of the N-propeptide of the hieroglyphic fibril, corrected for missing mass, is shown in Fig. 8B (solid line) together with a set of theoretical N-propeptide AMDs where the N-propeptides are confined to the overlap zone (dashed lines). As for the EDS skin fibrils, good agreement between the experimental and theoretical AMDs was obtained when E had a value of 70 × h (compare solid and center dashed line). Therefore, the total axial extent of the N-propeptides was 33 nm (70 × h = 20 nm for the globular domain and 45 × h = 13 nm for the triple helical domain).

DISCUSSION

In this study we have used quantitative dark-field STEM to investigate the conformation of N-propeptides in hieroglyphs and the near-cylindrical fibrils in the skin of people with EDS type VIIB. The hieroglyphic nature of the collagen/pNcollagenex co-polymers formed in vivo was explained by
confinement of N-propeptides to the fibril surface (Holmes et al., 1991b; Watson et al., 1992). Co-polymers of collagen and pNcollagen maintain a circular cross-section until the packing of N-propeptides at the fibril surface reaches a critical density. The fibril diameter at which departure from circularity occurs has been observed to be approximately 55 nm, in agreement with theoretical predictions where N-propeptides are located exclusively to the overlap zone (Holmes et al., 1991b). The results of the present study show that the occurrence of circular fibrils greater than 55 nm in diameter in tissues of individuals with EDS type VIIB is explained by the higher packing density of N-propeptide on the fibril surface permitted by two nonoverlapping axial locations of the N-propeptide (in gap and overlap zones). Doubling of the packing density of N-propeptides on the fibril surface doubles the maximum diameter for fibrils retaining circularity from 55 to 110 nm. The 92-nm diameter of the largest EDS skin fibril observed in the present study was, therefore, below the upper limit for circularity made possible by the location of N-propeptides to both gap and overlap zones.

The consequences of the absence of sequences encoded by exon 6 on the structure of procollagen are unknown. Previous studies of D-periodic assemblies of pNcollagen forming extended sheets showed N-propeptide occupying the overlap zone of the D-period in a bent-back conformation (Holmes et al., 1991a). In addition, rotary shadowing and electron microscopy of type I procollagen molecules from normal (Mould and Hulmes, 1987) and EDS type VIIB3 individuals shows that the bent-back conformation also occurs in procollagen molecules in solution. The results of the present study demonstrated, therefore, that the sequences encoded by exon 6 in COL1A2 are not essential for generating the bent-back conformation of the N-propeptide.

The ability of STEM to map the distribution of mass within fibril specimens together with comparison of experimental with theoretical data allowed an accurate estimate to be made of the axial extent of N-propeptides in the gap and overlap zones of EDS type VIIB fibrils. A special feature of the EDS type VIIB skin fibrils was that despite the range of conformations possible, N-propeptides were located exclusively to gap and overlap region with the absence of intermediate states. Theoretical and experimental observations showed that the axial extent of the N-propeptide was 33 nm regardless of the location of the N-propeptide to gap or overlap zones. (The value of the axial extent of the N-propeptide in hieroglyphic fibrils was also 33 nm.) Since the gap region of collagen fibrils is approximately 35 nm and the overlap is approximately 32 nm, N-propeptides in EDS type VIIB fibrils effectively cover overlap and gap zones. A value of 33 nm for the axial extent of the N-propeptide in the EDS fibrils and

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**Fig. 7.** Comparison of experimental and theoretical AMDs for the N-propeptide in EDS type VIIB skin fibrils. The experimental curve corrected to show the true N-propeptide AMD and smoothed to 15 nm resolution is shown as the solid line in B. Theoretical AMDs were calculated for different axial extents of the globular domain of the N-propeptide between 60 × h and 80 × h as described under “Materials and Methods” and “Results.” The theoretical AMDs for the two extreme values 60 × h and 80 × h are shown as long and short dashed lines, respectively. The theoretical AMD calculated with a value of 70 × h for the axial extent of the globular domain gave the best agreement with the experimental AMD and is shown as the medium dashed line. The axial arrangement of N-propeptides used to calculate the theoretical AMDs is shown schematically in A. Boxes 1 and 3, bent-back conformation. Boxes 2 and 4, extended conformation. TH represents the triple-helical domain of an N-propeptide; GD represents the globular domain. The alternative extents of the globular domain are indicated as broken lines.

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**Fig. 8.** Comparison of experimental and theoretical AMDs for the N-propeptide in hieroglyphic fibrils. The experimental curve is shown as the solid line in B. Theoretical AMDs were calculated for different axial extents of the globular domain of the N-propeptide between 60 × h and 80 × h. The theoretical AMDs for the two extreme values of 60 × h and 80 × h are shown as long and short dashed lines, respectively. The theoretical curve calculated with a value of 70 × h for the axial extent of the globular domain is shown as the medium dashed line. The axial arrangement of the N-propeptide in the bent-back conformation used to calculate the theoretical AMDs is shown schematically in A. TH, triple helical domain; GD, globular domain. The alternative extents of the globular domain are indicated as broken lines.
overlap zones in collagen fibrils may help to explain the joint hypermobility and other soft tissue complications observed in individuals with EDS type VIIIB. For example, a number of connective tissue components bind to collagen and can affect the rate of fibril formation (Vogel et al., 1984; Hedbom and Heinegärd, 1989). Of special relevance, decorin is known to bind directly to the d-staining band in the gap region of collagen fibrils (Scott and Orford, 1981). The presence of the N-propeptides could mask the binding sites for these molecules. The disruption of the binding of small proteoglycans and other matrix molecules or ectopic binding of extracellular matrix molecules to N-propeptides could have a pronounced effect on the mechanical properties of connective tissues.

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