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Physical and Chemical Properties of Human Type III Procollagen†

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ABSTRACT: Type III procollagen was isolated from the serum-free culture media of human foreskin fibroblasts by adsorption to controlled-pore glass beads and chromatography of the eluted procollagen pool on diethylaminoethylcellulose [Gerard, S., & Mitchell, W. M. (1979) *Anal. Biochem. 96*, 433–447]. Sodium dodecyl sulfate ($NaDodSO_4$) electrophoresis in 1% agarose–2% acrylamide gels with or without prior sample reduction revealed the predominance of a band with retarded mobility as compared to human procollagen I [hupro(I)]. Digestion of hupro(III) with pepsin yielded a product whose electrophoretic mobility was retarded for both the intact trimer and its reduced monomeric subunit as compared to that for the respective bands of rat skin (type I) collagen. $NaDodSO_4$–polyacrylamide gel electrophoresis of bacterial collagenase-digested hupro(III) demonstrated disulfide-bonded propeptides which upon reduction were replaced by two distinct monomeric propeptide bands. The amino acid composition of hupro(III) was similar to that of hupro(I) but contained increased amounts of HO–Pro and Cys and less Thr, Ala, Val, and Arg. Sedimentation equilibrium analysis in 1 M $CaCl_2$ yielded at extrapolated zero concentration a M_r of 505 ± 25K. A [hupro(III) – collagen(III)] circular dichroic difference spectrum suggests approximately 10% α helix. The zero-order mutarotation rate of hupro(III) ($v_0 = 55.0 \times 10^{-5}$ s^{-1}) was twice that of hucol(III) ($v_0 = 25.4 \times 10^{-5}$ s^{-1}) at 20 °C, which may reflect the influence of the interchain disulfide-bonded carboxyl propeptides on the process of collagen fold formation.

Type III collagen has been referred to as the fetal collagen, since it is relatively most predominant in the skin during fetal life (Epstein, 1974). However, this genetic type is also predominant in the arterial wall, intestine, and muscle and generally codistributes elsewhere in diminished relative proportion with type I collagen, excluding bone and tendon [for reviews, see Eyre (1980), Bornstein & Traub (1979), and Prockop et al. (1979)]. Type I and type III collagen, which together represent the majority of the body's interstitial collagen, are chemically similar, but type III is distinguished by about one-third more hydroxyproline and the presence of interchain disulfide bonds at the carboxyl terminus of the triple helical sequences (Chung et al., 1974; Glanville et al., 1976). Fibrils prepared in vitro from type I and type III collagens (Wiedemann et al., 1975) display a similar periodicity, although the cross-sectional diameter of type I appears to be greater than that of type III. This is consistent with the identification of type III collagen as one of the macromolecular constituents of the thin reticulin fibers which are seen in liver and spleen (Nowack et al., 1976a).

Procollagen represents the soluble biosynthetic precursor to the collagen molecule. Though most of the studies to date detailing procollagen structure have come from nonhuman systems, it appears that types I and III procollagen are very similar, both containing trimeric propeptides at each end which include a short collagen sequence in the amino terminus [for a review, see Timpl & Glanville (1981)] and an interchain disulfide-bonded structure at the carboxyl end [for reviews, see Eyre (1980), Bornstein & Traub (1979), Prockop et al. (1979)]. Type III procollagen, but not type I, however, contains additional interchain disulfide bonds between the amino propeptides (Bruckner et al., 1978; Fessler & Fessler, 1979; Nowack et al., 1976b; Uitto et al., 1980).

During intracellular procollagen biosynthesis, three separate precursor chains presumably associate in proper alignment such that subsequent formation of the triple helix ensues [for reviews, see Fessler & Fessler (1978) and Prockop et al. (1979)]. Since helical folding in vitro of free collagen chains in solution proceeds very slowly and incompletely (Altgelt et al., 1961), it was postulated by Speakman (1971) that the precursor sequences might function as "registration peptides" to facilitate the initial alignment of the three separate polypeptide chains and, thereby, subsequent formation of the triple helix. Although it was originally suggested that these registration peptides were located in the amino end of the proα[1]

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[1] Abbreviations: DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; CD, circular dichroism; $NaDodSO_4$, sodium dodecyl sulfate; hupro(III), human type III procollagen; hucol(III), human type III collagen; proα, collagen precursor chain containing amino and carboxyl propeptides [since only one type of chain has been identified to date for type III collagen, we have dropped the "1" designation in reference to proα(III) or α(III) chains]; proγ, three proα chains interchain linked by disulfide bonds; pγ, proteolytically altered proγ chains missing propeptides on one end; pN-collagen, pγ containing amino but not carboxyl propeptides.

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chains (Brownell & Veis, 1976; Fessler, 1974), more recent studies on type I and III procollagen have indicated that initial chain registration occurs between the carboxyl propeptides [for reviews, see Fessler & Fessler (1978) and Prockop et al. (1979) and also Bächinger et al. (1981) and Fessler et al. (1981)]. By direct physical analysis of mutarotation rates in human procollagen I, we were able to demonstrate that disulfide bonds, including the interchain bonds in the carboxyl propeptides, were necessary for an optimum rate of collagen helix formation in vitro (Gerard et al., 1981). A similar relationship has been previously described for bovine collagen III (Bächinger et al., 1980; Fujii & Kühn, 1975). In this study, we have extended our analysis of collagen fold formation to human procollagen III as well as a characterization of its major chemical and physical properties. Our data are consistent with the recent model of procollagen assembly proposed by Bächinger et al. (1981).

Materials and Methods

Procollagen Isolation. Type III procollagen was isolated from conditioned culture medium of human foreskin fibroblasts by glass bead and DEAE-cellulose chromatography exactly as outlined previously (Gerard & Mitchell, 1979). Briefly, confluent fibroblast cultures in glass roller bottles (1200-cm^2 surface area) were incubated for 24 h in 55-mL of serum-free minimal essential medium (Gibco) which contained 2.2 g/L $NaHCO_3$, 20 mM Hepes [*N*-(2-hydroxyethyl)piperizine-*N'*-2-ethanesulfonic acid] (Sigma), 50 units/mL penicillin, and 50 μg/mL each of streptomycin, ascorbate, and β-aminopropionitrile–fumarate. Harvested conditioned medium was adjusted to 25 mM EDTA and stored frozen at −20 °C. Isolation and purification of procollagen was performed on 5-L batches of accumulated conditioned medium, and the fractions from DEAE-cellulose chromatograms representing type III procollagen were pooled, dialyzed against buffer containing 50 mM Tris, pH 8.2 measured at 4 °C, 0.5 M NaCl, and 2.5 mM EDTA, and frozen for storage at −20 °C. The fraction of total procollagen represented by type III from DEAE-cellulose preparations ranged from 10 to 20%. Approximately 7 mg of purified type III procollagen, pooled from three 5-L preparations, was obtained for analysis.

Analytical Ultracentrifugation. Type III procollagen in 1 M $CaCl_2$ and 50 mM Tris-HCl, pH 7.6 measured at room temperature, was diluted to concentrations between 0.060 and 1.2 mg/mL and loaded into multisector Yphantis cells. The Spinco Model E analytical ultracentrifuge employed was equipped with a switched laser interference optical system (Williams, 1978). Sedimentation equilibrium analysis was performed by using the high-speed meniscus depletion method (Yphantis, 1964). The run was conducted at 10000 rpm with the temperature maintained at 23 °C. For each initial cell concentration, the apparent, cell-average molecular weight was evaluated by the relationship

$$M_{w,app} = \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \frac{d \ln c}{dr^2} \quad (1)$$

in which R is the gas constant, T is the absolute temperature, $\bar{v}$ is the partial specific volume of the protein, ρ is the solution density, ω is the angular velocity in radians per second, c is the concentration in fringes, and r is the radial distance in centimeters. The solvent density, ρ, was obtained by weighing 50 mL of the final procollagen dialysate in a volumetric flask, by means of a Mettler microbalance. The partial specific volume, $\bar{v}$, for type III procollagen (0.696 g/mL) was estimated from the amino acid composition (Cohn & Edsall, 1943).

Limited Pepsin Digestion. Type III procollagen (2 mL, 125 μg/mL) dialyzed into 0.5 M acetic acid was incubated for 24 h at 4 °C after adding 5 μg of pepsin (Worthington/Millipore) in 5 μL of 0.5 M acetic acid. Digestion was stopped by dialysis at 4 °C against buffer containing 0.5 M NaCl, 50 mM Tris-HCl, pH 8.2, and 0.1 mM EDTA.

Circular Dichroism. Sample preparation, apparatus, and data analysis were performed as described previously where applicable (Gerard et al., 1981). All samples were in buffer containing 0.5 M NaCl, 50 mM Tris-HCl, pH 8.2 measured at 4 °C, and 0.1 mM EDTA. Spectra were normalized to mean residue specific ellipticity, [Θ], which has units of deg·cm^2/dmol, by using eq 2, in which Θ is the corrected

$$[\Theta] = \Theta(MRW)/(10lc) \quad (2)$$

ellipticity in mdeg, MRW is the mean residue weight, l is the pathlength in cm, and c is the concentration in mg/cm^3. A mean residue weight of 97.4 was calculated for type III procollagen from the amino acid composition. For human type III collagen, values assumed were a MRW of 91.2 and molecular weight of 92400 for α1(III) (Chung et al., 1974). The number of residues per mole of total type III propeptides was estimated from the molecular weights and mean residue weights of human type III collagen and procollagen. Refolding rates were examined subsequent to heating to 45 °C for 20 min and then quench cooling to 20 °C. The helix fraction, f, was calculated from the ellipticities at 223 nm by using eq 3

$$f = (\Theta_t - \Theta_{45})/(\Theta_{20} - \Theta_{45}) \quad (3)$$

where Θ_t is the ellipticity at time t, Θ_{45} is the ellipticity at 45 °C, and Θ_{20} is the ellipticity at 20 °C prior to heating. The magnitude of the temperature-induced transition at 223 nm, $\Delta[\Theta]_{223}$, was determined according to eq 4:

$$\Delta[\Theta]_{223} = (\Theta_{20} - \Theta_{45})(MRW)/(10lc) \quad (4)$$

When applicable, first-order rate constant were calculated based on the slope of the semilog plot described by eq 5. Zero-order rate constants, v_0, were taken as the slope of f plotted against t.

$$-\ln (1 - f) = kt + \text{constant} \quad (5)$$

The CD difference spectrum for type III (procollagen − collagen) was estimated by first converting spectra from units of mean residue to molar [Θ], taking the difference, and then converting back to units of mean residue [Θ]. Estimates of propeptide secondary structure were made by assuming (1) the specific ellipticities reported by Chen et al. (1974) for α helix, β structure, and aperiodic structure, (2) that 224 nm represents a crossover point for both β and aperiodic structure such that only α helix and collagen helix would contribute ellipticity at this wavelength, and (3) that 214 nm represents a crossover point for aperiodic structure, thereby limiting CD contributions at this wavelength to α helix, β structure, and collagen helix.

Other Procedures. Amino acid analysis, bacterial collagenase digestion, $NaDodSO_4$–acrylamide gel electrophoresis, and protein estimation were performed essentially as described previously (Gerard et al., 1981). The agarose–acrylamide–$NaDodSO_4$ electrophoresis procedure (Gerard & Mitchell, 1979) was modified by increasing the running current to 9 mA/gel, which permitted completion of the run in 3 h. For type III procollagen, the correction factor for tryptophan fluorescence protein estimates was 2.44, based on fluorescamine (Roche) assay of hydrolysates. Estimation of the type III collagen concentration was performed by amino acid analysis, using published data for reference (Chung et al.,

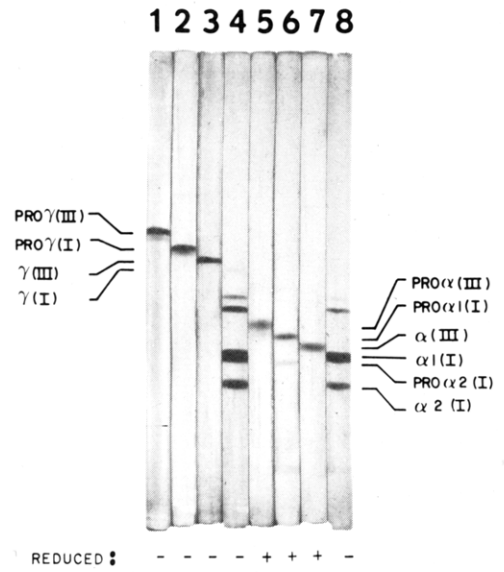

FIGURE 1: $NaDodSO_4$–agarose–polyacrylamide gel electrophoresis of hupro(III) and hucol(III). Gels contained 2% acrylamide and 1% agarose. Migration is from top to bottom. Samples for gels 5–7 were reduced with 1% β-mercaptoethanol prior to electrophoresis. Gels 1 and 5, hupro(III); gels 2 and 6, hupro(I); gels 3 and 7, hucol(III); gels 4 and 8, lathrytic rat skin (type I) collagen.

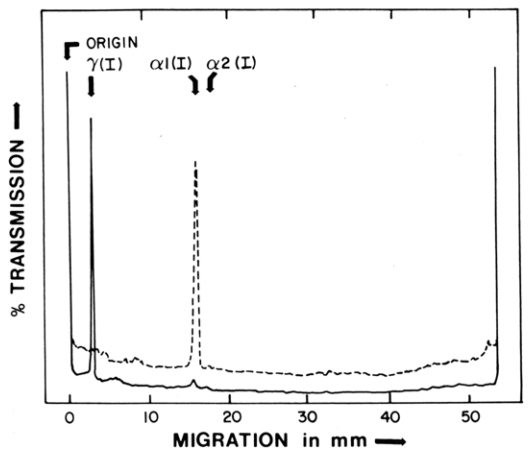

FIGURE 2: Five percent $NaDodSO_4$–polyacrylamide gel electrophoresis of hucol(III). The negative of a Coomassie Blue stained slab gel was scanned for percent transmission by using white light. Migration is from left to right. Unreduced sample is plotted as a solid line, and sample reduced with 1% β-mercaptoethanol prior to electrophoresis is plotted as a broken line. Labeled arrows denote positions of rat skin type I collagen standards.

1974). Scanning of size-120 negatives of Coomassie Blue stained 5% $NaDodSO_4$–polyacrylamide slab gels was performed on a Beckman DU-8 spectrophotometer in the percent transmission mode by using white light (i.e., undispersed radiation). Coomassie Blue stained agarose–acrylamide gels of unreduced hupro(III), which had been electrophoresed for an additional 1.5 h to increase band separation, were scanned for OD at 550 nm on the same instrument, which directly integrated peak areas on OD plots. Further estimation of relative peak areas was achieved by Gaussian approximation using the Marquardt algorithm (Marquardt, 1963). $NaDodSO_4$–polyacrylamide electrophoresis of bacterial collagenase-digested type III procollagen was performed on a 5–20% gradient slab. Riboflavin (5 μg/mL) was included in the light solution for polymerization, and the stacking gel contained 3% acrylamide. Electrophoresis was conducted at 25-mA constant current for 13 h, and the slab was stained in 10:10:80 methanol–acetic acid–water which contained 0.05% Coomassie Blue. Other details of electrophoretic procedures were as described previously (Gerard et al., 1981). Collagenase-digested samples and standard proteins for electrophoresis were reduced at 100 °C for 5 min in digestion buffer adjusted to 1% $NaDodSO_4$ and 12 mM β-mercaptoethanol and then alkylated in the dark at room temperature for 15 min in the presence of 25 mM iodoacetamide. Following addition of the equivalent amount of β-mercaptoethanol, samples were dialyzed against sample buffer at room temperature, as described previously (Gerard & Mitchell, 1979).

Results

Electrophoretic Profiles of Procollagen and Collagen. The electrophoretic profiles of hupro(III) and hucol(III), as resolved by the $NaDodSO_4$–agarose–polyacrylamide composite gel system, are shown in Figure 1. For comparison, Figure 2 demonstrates the electrophoretic profiles of hucol(III) with respect to type I collagen standards in a conventional 5% $NaDodSO_4$–polyacrylamide gel system.

In the agarose–polyacrylamide gel system, hupro(III) is represented by the predominance of a single trimer band in the unreduced condition, proγ(III) (Figure 1, gel 1), which migrates more slowly than the proγ(I) trimer band of hupro(I) as well as the aldehyde cross-linked γ(I) chain of rat skin collagen (Figure 1, gels 2 and 4, respectively). The unreduced hucol(III) trimer band, γ(III) (Figure 1, gel 3), also displays a retarded mobility with respect to γ(I) (Figure 1, gel 4). Similar relationships are observed for the reduced monomer bands. Proα(III) (Figure 1, gel 5) is seen to migrate more slowly than proα1(I) and α1(I) (Figure 1, gels 6 and 8, respectively), and the mobility of α(III) (Figure 1, gel 7) is correspondingly retarded as compared to that of α1(I) (Figure 1, gel 8).

While the differences in mobility described in Figure 1 are small, they were found to be reproducible, if the precautions outlined previously (Gerard & Mitchell, 1979) were taken. In addition, the order of the relative mobilities was verified by agarose–polyacrylamide slab gel electrophoresis (not shown), though the resolution achieved was suboptimal as compared to that of the cylindrical gels shown in Figure 1.

$NaDodSO_4$ electrophoretograms of pepsinized hupro(III) in conventional 5% acrylamide slab gels (Figure 2) demonstrated that essentially all recovered material was represented by type III collagen. Unreduced material migrated as a single band which comigrated with the γ(I) band of rat skin collagen. Upon reduction, this band was replaced by a more rapidly migrating band which comigrated with the α1(I) band of rat skin collagen.

A minor band in gel 1 of Figure 1 of mobility intermediate to proγ(III) and proγ(I) (Figure 1, gels 1 and 2, respectively) is thought to represent a small amount of a partially degraded type III procollagen intermediate. Small quantities of type I procollagen, amounting to 5% based on integration of OD_{550} scans of $NaDodSO_4$–agarose–polyacrylamide gels, were also present in the DEAE-cellulose-purified type III procollagen. While the minor band in gel 1 could not be reliably estimated by direct integration of OD scans due to its proximity to proγ(III), estimation by Gaussian approximation suggested about 20% gel 1 minor band and 74% proγ(III), based on an average of four separate gel scans. This analysis also confirmed spectrophotometer-integrated estimates of 5–6% proγ(I) in the hupro(III) preparation.

Identification of Bacterial Collagenase-Resistant Peptides in Hupro(III). Electrophoretic profiles of bacterial collagenase-digested hupro(III) and hucol(III), as resolved by a

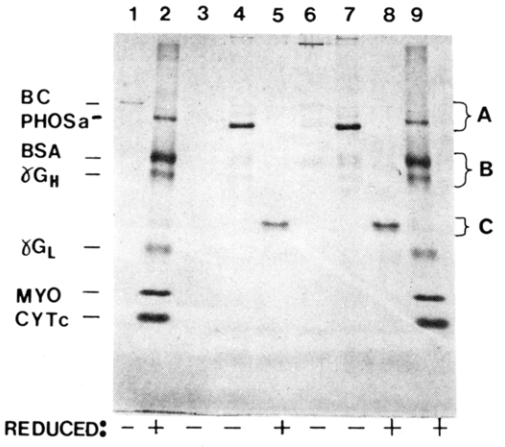

FIGURE 3: $NaDodSO_4$–polyacrylamide gel electrophoresis of bacterial collagenase-digested hupro(III) and hucol(III). Electrophoresis was performed on a 5 to 20% gradient slab gel, with a 3% stacking gel. Track 1, bacterial collagenase (BC); tracks 2 and 9, standard proteins: phosphorylase *a* (PHOSa), bovine serum albumin (BSA), γG heavy chain (γG_H), γG light chain (γG_L), myoglobin (MYO), and cytochrome *c* (CYTc); track 3, digested rat skin collagen; tracks 4 and 7, unreduced digested hupro(III); tracks 5 and 8, reduced and alkylated digested hupro(III); track 6, unreduced digested hucol(III).

5–20% gradient slab gel, are shown in Figure 3. In the unreduced condition, digested hupro(III) reveals several bands which migrate in the regions labeled A and B (Figure 3, tracks 4 and 7). Three bands are present in the A region, which corresponds to a molecular weight of 90–100K, based on globular standards (Figure 3, tracks 2 and 9). Of these three bands, the one closest to the origin is observed to comigrate with bacterial collagenase (Figure 3, track 1), and this band is also seen in the reduced condition (Figure 3, tracks 5 and 8). Of the remaining two bands seen in region A, the most rapidly migrating band is clearly the most predominant. A faint band comigrating with this band is seen in the digested hucol(III) sample (Figure 3, track 6). In the digested hupro(III) sample, the somewhat more diffuse bands seen in region B correspond to a molecular weight range of 45–65K with respect to globular standards. Upon reduction, essentially all material in regions A and B is replaced by two closely spaced but distinctly resolved bands which migrate in the region labeled C (Figure 3, tracks 5 and 8). A molecular weight of approximately 35K would be predicted for these bands, based on globular standards. None of the bands described above which were observed in electrophoretic profiles of either unreduced or reduced samples of digested hupro(III) were seen in $NaDodSO_4$ electrophoretograms of undigested hupro(III) (not shown). Other faint bands seen in Figure 3 include undigested γ(III) and proγ(III) near the origin (tracks 6 and 7, respectively).

Amino Acid Composition. The results of hupro(III) amino acid analysis are shown in Table I. Our analysis is qualitatively similar to those previously reported for type III procollagen isolated from other species (Timpl et al., 1975; Lenaers & Lapiere, 1975; Byers et al., 1974). Though hupro(III) is similar in composition to hupro(I) (Table I), noteworthy differences include 28% more hydroxyproline, 22% less threonine, 24% more serine, 14% less alanine, 35% less valine, 42% more cysteine, 12% less arginine, and slightly more acidic residues. The differences for Hyp, Thr, Ala, Val, and Arg are consistent with quantitatively similar compositional differences between human types I and III collagens (Table I). The increased cysteine content reflects in part the two extra residues present in the α(III) chain (Table I) but presumably also an increased content of this residue in the type III propeptide

Table I: Amino Acid Composition of Human Procollagens and Collagens in Residues per 1000

residue	pro(III)[a]	pro(I)[b]	col(III)[c]	col(I)[d]
Hyp	95	74	125	92
Asp	71	64	42	42
Thr	21	27	13	17
Ser	51	41	39	36
Glu	92	85	71	69
Pro	103	108	107	128
Gly	258	262	350	336
Ala	83	97	96	113
½-Cys	17[e]	12	2	
Val	22	34	14	24
Met	4.5[f]	8	8	6
Ile	19	19	13	9
Leu	33	34	22	24
Tyr	11	13	3	2.5
Phe	22	22	8	12
Hyl	7	7	5	6
His	10	8	6	5
Lys	36	35	30	27
Arg	44	50	46	50
Trp	ND[g]	ND		

[a] Values represent a composite of seven analyses, uncorrected for hydrolytic loss, and for which the average coefficient of variation was 8%. [b] As taken from Gerard et al. (1981). [c] As taken from Chung & Miller (1974). [d] Calculated from Epstein et al. (1971). [e] Determined as cysteic acid. [f] Determined as methionine sulfone. [g] Not determined.

sequences. As noted previously for human type I procollagen (Gerard et al., 1981), the deviation in overall composition of hupro(III) from that of hucol(III) (Table I) reflects the contribution of the propeptide sequences. Though tryptophan was not quantitated in our analyses, the tryptophan fluorescence correction factor for hupro(III) determined from fluorescamine (Roche) assay of hydrolysates was within 6% of that factor previously determined for hupro(I) (Gerard et al., 1981), which suggests a similar tryptophan content for the human type I and type III collagen precursors.

Molecular Weight Determination by the Meniscus Depletion Method of Sedimentation Equilibrium. Analytical ultracentrifugation of hupro(III) was performed on an instrument equipped with a pulsed-laser interference optical system, which yielded fringe patterns of superior resolution as compared to those obtained with the conventional Spinco light source. For minimization of aggregation effects as well as reduction of the time to equilibrium, 1 M $CaCl_2$ was chosen as the solvent system, as employed previously for the tadpole collagenase fragments of rat skin collagen (Kang et al., 1966). Equilibrium was achieved by 72 h at 10 000 rpm, based on comparison of fringe patterns taken at 12-h intervals. Downward curvature of ln *c* vs. r^2 plots was observed for the data corresponding to the highest concentrations. This effect was attributed to effects of "Wiener skewing" (Svensson, 1954; Williams, 1978), and this portion of the plots was disregarded for the purposes of calculating the apparent molecular weight at the initial cell concentration (C_0). Extrapolation to zero initial cell concentration yielded a molecular weight for the intact trimer of 505 000 ± 25 000 (95% confidence limits, based on the SD of the *y* error).

CD Spectra of Hupro(III) and Hucol(III). Figure 4 and Table II show the CD spectra and values at 223 nm, respectively, for hupro(III) and hucol(III) in both the native and unfolded conformations (20 and 45 °C). The observed spectra are characteristic for collagen-containing proteins in that they demonstrate a positive CD extremum at 223 nm for the 20 °C condition which is replaced by a negative trough for the unfolded conformation at 45 °C (Tiffany & Krimm, 1969).

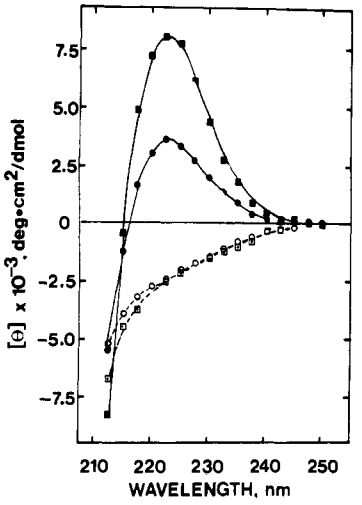

FIGURE 4: CD spectra of hupro(III) and hucol(III). All samples were in buffer containing 0.5 M NaCl, 50 mM Tris-HCl, pH 8.2 at 4 °C, and 0.1 mM EDTA. Data plotted represent an average of four spectra of hupro(III) (two scans each at 67 and 123 μg/mL) and two spectra of hucol(III) (60 μg/mL). Hupro(III) at 20 °C (●) and at 45 °C (○); hucol(III) at 20 °C (■) and at 45 °C (□).

Table II: CD Values of Human Type III Procollagen and Collagen

sample	μg/mL	$[\Theta]_{223}$[a] 20 °C	$[\Theta]_{223}$[a] 45 °C	$\Delta[\Theta]_{223}$[a]
procollagen III	124	3600	−2400	6000
collagen III	60	8000	−2450	10450

[a] Units are deg·cm²/dmol.

As compared to the CD values reported previously for hupro(I) [Gerard et al (1981), Table III], the hupro(III) spectra are characterized by a somewhat greater magnitude of specific ellipticity at 223 nm (Table II) and thereby a 43% greater transition magnitude, $\Delta[\Theta]_{223}$. This may reflect the 37% larger $\Delta[\Theta]_{223}$ observed for hucol(III) (Figure 4; Table II) as compared to that for rat skin (type I) collagen (Gerard et al., 1981).

The CD difference spectra of hupro(III)–hucol(III), representing total type III propeptide secondary structure, were calculated from the 20 and the 45 °C data. The data used represented averages of four spectra of hupro(III) and of two spectra of hucol(III), from which standard deviations were calculated for each point. The difference spectra calculated for the two temperatures were nearly identical in that any differences between them fell within the range specified by the pooled standard deviation calculated for each temperature. This indicates that in the intact proγ(III) molecule, there is negligible change in propeptide secondary structure over the temperature range of 20–45 °C, as observed by CD difference spectroscopy.

The difference spectrum shown in Figure 5, which represents an average of 20 and 45 °C spectra, is characterized by a negative trough in the region of 210–230 nm. While signal-to-noise ratios on the spectropolarimeter prevented measurements below 210 nm, it was possible to predict an α-helical content of 9 ± 1% (pooled SD) for the total propeptide secondary structure, based on $[\Theta]_{224}$ (see Materials and Methods).

Collagen Fold Re-formation by Hupro(III) and Hucol(III). The time course and associated rate constants of collagen helix reformation at 20 °C by hupro(III) and hucol(III) are shown in Figure 6 and Table III, respectively. In both cases, renaturation was observed to proceed in three phases, as described previously for hupro(I) (Gerard et al., 1981): (1) an initial

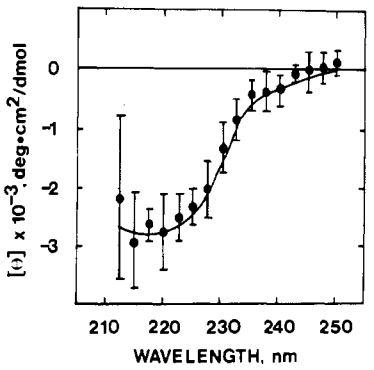

FIGURE 5: CD difference spectrum of hupro(III)–hucol(III). The difference was calculated from the spectra shown in Figure 4 as described under Materials and Methods. Error bars represent the pooled standard deviations calculated from the hupro(III) and hucol(III) spectra for each point.

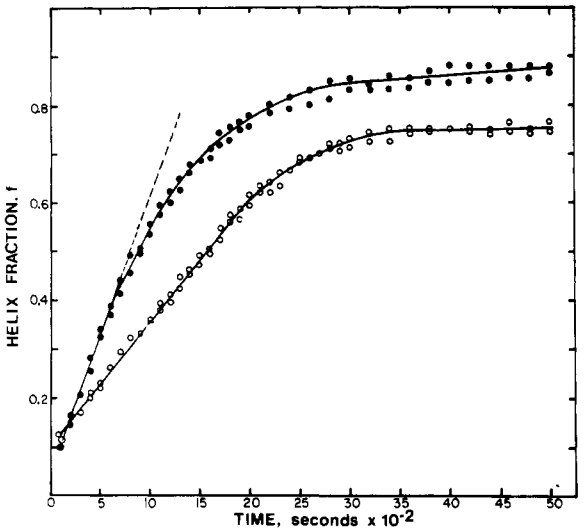

FIGURE 6: Refolding of hupro(III) and hucol(III) at 20 °C. All samples were in buffer containing 0.5 M NaCl, 50 mM Tris-HCl, pH 8.2 at 4 °C, and 0.1 mM EDTA. For each sample, ellipticity at 223 nm was recorded at 20 °C and then at 45 °C after 5 min at this temperature. After 20 min at 45 °C, sample was quench-cooled to 20 °C and ellipticity was followed as a function of time. Hupro(III) (●), two samples at 123 μg/mL; hucol(III) (○), two samples at 60 μg/mL. The broken line represents an extrapolation of the slope calculated from the 100–600-s range of the hupro(III) plot.

Table III: Collagen Refolding Rates at 20 °C

condition	μg/mL	time range (s)	zero order, $10^5 v_0$ (SD) (s^{-1})	first order, $10^5 k$ (SD) (s^{-1})
collagen III	60	100–2000	25.4 (0.3)	
procollagen III	67	100–600	52.9 (2.0)	
		100–1500		78.3 (2.8)
	123	100–600	55.8 (1.9)	
		100–1500		77.3 (1.0)
procollagen I[a]	72	120–1500		38.1 (1.0)

[a] Taken from Gerard et al. (1981).

rapid phase in the first 60–80 s [t zero taken from the beginning of the 45–20 °C temperature shift as described in Gerard et al. (1981)], representing 8–10% of the initial helicity, characterized by a first-order rate constant of (1.4–1.6) × 10^{-3} s^{-1}; (2) a second and major refolding phase from approximately 100 to 2000 s, accounting for most of the refolded structure formed over the time period examined; (3) a subsequent period during which time the refolding rate tapered off. As was reported for hupro(I) (Gerard et al., 1981), hupro(III) was observed to refold by an apparent first-order mechanism for

the major phase, based on the linearity of $-\ln(1-f)$ vs. t plots (not shown), at a rate approximately twice that previously observed for hupro(I) (Table III). In contrast, hucol(III) appeared to refold by a zero-order process over the major refolding phase, with the helix fraction plotting linearly over time (Figure 6), at a rate of only half that estimated for hupro(III) (Table III). Hupro(III) regained approximately 87% of the initial helicity by the end of the 5000-s time period examined (Figure 6), whereas a lesser value of 75% was achieved by hucol(III) over this time course (see Discussion).

As shown previously for hupro(I) (Gerard et al., 1981), the experimental conditions employed for collagen refolding studies had negligible effect on sample stability, as indicated by near identical $NaDodSO_4$ electrophoretic profiles generated from hupro(III) and hucol(III) before and after experimental manipulation (not shown).

Discussion

Isolation of procollagen from human fibroblast tissue culture medium is efficiently and rapidly accomplished by adsorption to and differential elution from controlled-pore glass beads (Gerard & Mitchell, 1979). Normally, type I accounts for most of the procollagen in human fibroblast culture medium (Lichtenstein et al., 1975; Uitto et al., 1980). Since glass bead chromatography facilitated repeated extractions of large volumes of conditioned medium, however, milligram quantities of type III procollagen were readily accumulated from serial DEAE-cellulose preparations. Moreover, the use of this method appears to preclude isolation of other components which copurify with type III procollagen when isolated by other methods (Burke et al., 1977; Uitto et al., 1980).

We have established the type III genetic identity of this procollagen based on the following criteria: (1) $NaDodSO_4$ electrophoretic profiles of either intact or pepsinized material revealed the predominance of a single high molecular weight band which upon reduction was replaced by a single band migrating in the proα or the α chain region of the gel, respectively; (2) the relative concentrations and position of the material in DEAE-cellulose chromatograms [Gerard & Mitchell (1979), Figure 2] is characteristic for type III procollagen isolated from human fibroblast culture medium (Lichtenstein et al., 1975) or from other nonhuman sources (Church et al., 1973; Smith et al., 1977; Burke et al., 1977); (3) the amino acid composition with respect to hupro(I) reflects characteristic differences between the reported amino acid compositions for human types I and III collagens [Epstein et al. (1971) and Chung & Miller (1974), respectively].

As shown previously (Gerard & Mitchell, 1979), type III procollagen displays a retarded electrophoretic mobility with respect to type I procollagen in the $NaDodSO_4$–agarose–polyacrylamide gel system. Here, we demonstrate that type III collagen also migrates more slowly in this gel system as compared to type I collagen. Since an increased content of hydroxylated residues was previously shown to be associated with retarded electrophoretic mobility (Crouch & Bornstein, 1978; Sage et al., 1979), the elevated levels of hydroxyproline in hucol(III) and hupro(III) may contribute to this effect. Though small differences between the electrophoretic mobilities of type I and type III collagen or procollagen have been observed on polyacrylamide gels (Peltonen et al., 1980; Sykes et al., 1976), the distinct electrophoretic mobility differences seen in the agarose–polyacrylamide gel system are in contrast to the usual near comigration of these bands in conventional $NaDodSO_4$–polyacrylamide gel systems (Figure 2; Blackwell & Bensusan, 1977; Burke et al., 1977; Byers et al., 1974; Fessler et al., 1981; Lichtenstein et al., 1975; Sodek & Limeback, 1979; Timpl et al., 1975). These differences in electrophoretic mobility on agarose–polyacrylamide gels suggest a simplified alternative to existing methods for the relative quantitation of type I and type III procollagen (Uitto et al., 1980). In particular, estimates based on the separation of type I and type III collagen chains in pepsinized material may significantly underestimate the relative proportion of type III present (Burke et al., 1977; Uitto et al., 1980).

As previously described (Gerard & Mitchell, 1979; Goldberg, 1977), we did not identify any free α- or β-chain partially processed intermediates in our preparations of hupro(III) isolated from fibroblast culture medium. The minor band in gel 1 of Figure 1 may represent a pγ(III) intermediate. An electrophoretic mobility intermediate to that of proγ(III) and γ(III) and its conversion to γ(III) subsequent to limited proteolysis by pepsin are both consistent with this interpretation. Alternatively, the retarded mobility of this band with respect to that of proγ(I) might argue against this identification, since one might expect the truncated type III precursor to migrate more rapidly in $NaDodSO_4$ gels than intact type I procollagen, on the basis of molecular size. This consideration suggests that the minor gel 1 band could represent a truncated proγ(III) form in which only a portion of one propeptide has been removed. Since we have not further characterized this intermediate, we cannot differentiate between these two possibilities. Regardless, we believe that the above considerations and the described behavior of type I and III collagens in the composite agarose–acrylamide gel system justify the identity of a truncated proγ(III) intermediate for this minor band.

Electrophoretic profiles of bacterial collagenase-digested hupro(III) (Figure 3) demonstrated several bands in the unreduced condition migrating in regions A and B which upon reduction were replaced by a more rapidly migrating closely spaced doublet in region C. Optimum resolution of the reduced doublet required alkylation prior to electrophoresis. This behavior of intrachain disulfide-bonded proteins has been previously described (Lane, 1978). Since these bands were not seen in profiles of intact hupro (III), we presume that they represent bacterial collagenase-resistant human type III propeptides. Although we have not specifically identified the amino and carboxyl propeptide bands in our system, the doublet observed under reducing conditions closely resembles the electrophoretic profiles of the reduced bacterial collagenase-resistant amino and carboxyl propeptides of chick procollagen III described by Fessler & Fessler (1979). Similarly, the $NaDodSO_4$ electrophoretic mobilities of the unreduced bands in regions A and B approximate those previously described for the unreduced chick type III carboxyl and amino propeptides, respectively (Bächinger et al., 1981; Fessler et al., 1981). While the demonstration of the doublet bands under reducing conditions is consistent with the presence of amino and carboxyl propeptides in hupro(III), this identification by analogy to the chick system is not definitive and these bands may or may not represent the intact propeptides (see below).

Sedimentation equilibrium analysis for the purpose of molecular weight estimation of intact procollagen has rarely been employed. Although this method has been used to estimate the mass of isolated nonhuman propeptides (Engel et al., 1977; Olsen et al., 1977; Bruckner et al., 1978), we are aware of only one other such determination for the presumptive intact collagen precursor, which was previously reported from this laboratory (Bańkowski & Mitchell, 1973). While the human procollagen was not characterized as to its $NaDodSO_4$ elec-

trophoretic homogeneity or relative content of genetic types, our molecular weight estimate for hupro(III) in these studies is nonetheless in close agreement with that determined previously for human procollagen (Bańkowski & Mitchell, 1973). The sharp interference patterns obtained with the switched laser light source in the present study minimized ambiguities in pattern analysis. The presence of some truncated proγ(III) intermediate in our material may be responsible for some underestimation in our determination. Since we did not determine the mass of this intermediate, however, we did not correct for the potential bias in our analysis of the sedimentation equilibrium data.

Our mass estimate of 505 ± 25K for hupro(III) predicts a molecular weight of 160–175K for the human monomer proα(III) chain. This value represents a somewhat larger mass for proα(III) than has been estimated previously by using other methods (Anesey et al., 1975; Blackwell & Bensusan, 1977; Krieg et al., 1979; Lenaers & Lapiere, 1975; Lichtenstein et al., 1975). While our mass estimate might appear to be consistent with the sum of the monomer propeptide masses predicted by globular standards in $NaDodSO_4$ electrophoresis (i.e., approximately 35K each for the carboxyl and amino propeptides) and the reported mass of the α(III) chain (Chung et al., 1974), the isolated propeptides have previously been shown to migrate anomalously with respect to globular protein standards in $NaDodSO_4$ gel systems (Becker et al., 1976). Furthermore, our mass estimate for hupro(III) would imply a greater molecular size for the propeptide sequences than has been reported by sedimentation equilibrium for the calf type III amino and chick type I carboxyl propeptides (Bruckner et al., 1978; Olsen et al., 1977). $NaDodSO_4$ coelectrophoresis of calf, chick, and human propeptides would establish relative size relationships. However, nonhuman propeptide species were unavailable to us, and it is possible that despite the influence of 1 M $CaCl_2$, some aggregation of hupro(III) in the ultracentrifuge cell could have spuriously elevated the estimated mass. While this influence of sample heterogeneity typically results in upward curvature of ($\ln c$ vs. r^2) plots, which was not observed in our analysis, superimposed nonideality producing downward curvature of these plots could have conceivably masked the former effect, yielding spuriously high values for the mass of hupro(III). Alternatively, it is possible that procollagen mass estimates based on those of the isolated propeptides may underestimate the true size of the intact precursor secondary to covalent modification of these sequences during their isolation. This problem has historically hampered efforts to characterize procollagen.

The relationship between the hupro(III) and hucol(III) CD spectra was analogous to that previously described for type I procollagen and collagen (Gerard et al., 1981). We did not observe a red shift in the CD spectra of hupro(III) as previously reported by Peltonen et al. (1980). Our protein concentrations (67 and 123 μg/mL) were apparently considerably greater than those employed by Peltonen et al. (1980), which may be related to the difference between observed CD spectra.

From the human type III (procollagen − collagen) CD difference spectrum, we were able to estimate 9 ± 1% α helix for the total type III propeptides. Efforts to quantitatively estimate the percent β structure were hampered by the relative size of the ±SD range of the CD difference spectrum at 214 nm (Figure 5) with respect to the smaller magnitude of $[\theta]_{214}$ for β structure (Chen et al., 1974). Studies on the bovine type III amino propeptide (Bruckner et al., 1978) indicated that it contains about 15 triplets of interchain disulfide-bonded collagen helix proximal to the major globular domain. A similar structure without interchain bonds has also been described for the type I amino propeptide isolated from chick (Pesciotta et al., 1980), calf (Hörlein et al., 1979), and sheep (Engel et al., 1977; Becker et al., 1976). Immunologic and chromatographic properties of presumptive propeptides in human ascitic fluid are consistent with similar structures for the human and bovine type III amino propeptides (Rohde et al., 1979). If we include the CD contributions of the propeptide collagen sequence in our calculations, this would increase our estimate of α helix to 11 ± 1.4% for the intact human type III total propeptides. The CD spectrum of the globular Col 1 fragment from the sheep type I amino propeptide suggested the possibility of substantial β structure (Engel et al., 1977), and similar results were reported for the analogous type III peptide fragment from calf (Bruckner et al., 1978). Given the limitation stated earlier, our analysis of the CD difference spectrum would predict an upper limit of 19% β structure in hupro(III), based on the mean $[\theta]_{214}$ + 1 standard deviation. In view of the imprecision of this estimate, however, we cannot definitively state that our data are consistent with the prior characterizations of the sheep and calf propeptides.

An estimate of about 10% α helix for hupro(III) propeptides is very similar to the estimate made by CD criteria for hupro(I) (Gerard et al., 1981). A more rigorous analysis of the CD difference spectrum was limited by an unfavorable signal-to-noise ratio below 212 nm. Nonetheless, this represents to our knowledge the first conformational analysis of human procollagen propeptide secondary structure.

Refolding of hupro(III) subsequent to thermal denaturation was similar to that previously described for hupro(I) under identical conditions (Gerard et al., 1981). During the major refolding phase, however, the first-order rate constant of mutaroation was twice that of hupro(I) (Table III). Hucol-(III) renaturation, in contrast, was characterized by apparent zero-order kinetics with an associated rate half that observed for hupro(III) (Table III). Our results are in apparent disagreement with those of Bächinger et al. (1980), who reported approximately equivalent zero-order rate behavior for the refolding of type III collagen and pN-collagen isolated from fetal calf skin. Although differences in experimental design (solvent) or basic refolding kinetics of bovine vs. human type III precursors may explain the variance in kinetic data, the lack of carboxyl propeptides in pN-collagen suggests significant influences by the carboxyl terminus of hupro(III) in secondary structure formation. Both type I and type III procollagen correctly form disulfide-linked trimeric structures under conditions which block proline hydroxylation and collagen helix formation (Fessler & Fessler, 1974; Harwood et al., 1977; Fiedler-Nagy et al., 1981; Fessler et al., 1981). Recent studies on chick pro(III) biosynthesis provide strong evidence that chain registration and subsequent collagen helix formation is preceeded by association of the carboxyl propeptides (Bächinger et al., 1981). Thus, the two-fold greater rate of refolding observed for hupro(III) as compared to hucol(III) may reflect the influence of the interchain disulfide-bonded type III carboxyl propeptides in the intact precursor, these propeptides being absent in pN-collagen(III). Moreover, the difference in rate orders [hupro(III) and hucol(III)] further suggests a more complex influence of the carboxyl propeptides on collagen helix formation.

Hupro(III) was observed to refold to approximately 87% helix fraction by the 5000-s experimental end point, which represents about 10–15% greater helicity than was previously observed for hupro(I) (S. Gerard, D. Puett, and W. M.

Mitchell, unpublished results; Bruckner & Prockop, 1981). The presence in type III procollagen of additional interchain disulfide bonds in the amino propeptides [for a review, see Timpl & Glanville (1981)] may contribute to this increased extent of refolding, since the type III precursor thereby has the additional amino-terminal end locked "in register" (Fessler & Fessler, 1979). The possibility that these amino propeptide disulfide bonds may additionally contribute to an enhanced initial refolding rate is unlikely since helix formation appears to initiate from the carboxyl terminus of the precursor (Bächinger et al., 1980).

Although we observed a somewhat lower yield of refolded hupro(III) as compared to that of calf pro(III) reported by Bächinger et al. (1980), our results are qualitatively in agreement with previous refolding studies on type III collagen and procollagen, in which generally high yields of refolded structure were reported, as observed by optical criteria (Fujii & Kühn, 1975; Byers et al., 1974). The near-identical NaDodSO$_4$ electrophoretograms of either hupro(III) or hucol(III) before and after experimental manipulation (not shown) argue against significant covalent modification during the experimental protocol, consistent with that described previously for hupro(I) (Gerard et al., 1981). Increased ratios of type I collagen if present, however, would tend to artifactually decrease the apparent refolding rate for hucol(III), since the unlinked type I chains would not undergo significant renaturation over this time course (Altgelt et al., 1961). Nonetheless, NaDodSO$_4$ gel profiles of hucol(III), as shown in Figures 1 and 2, suggested only minimal type I contamination and therefore minimize this contribution to the estimated hucol(III) refolding rate.

In our investigations of procollagen refolding, we have not characterized the fidelity of the refolded helical structure. Assay of accurately folded triple helical sequences by resistance to proteolytic digestion has recently been refined and recharacterized such that digestion can be completed within 2 min (Bruckner & Prockop, 1981). This improvement minimizes some of the potential problems associated with this approach, as have been previously discussed (Fessler & Fessler, 1978; Gerard et al., 1981; Kao et al., 1977). The use of this method in conjunction with thermal stability studies has suggested that whereas much of the helical structure in refolded type I procollagen may be misaligned or poorly annealed (Bruckner & Prockop, 1981), type III collagen or procollagen refolding yields helical structure with high precision (Bächinger et al., 1980). As suggested by Fiedler-Nagy et al. (1981), the deficit of precisely refolded end product formed under experimental conditions implies that one or more unknown physiologic factors, which are absent from experimental systems, would otherwise mediate the formation of more precise helical structure in vivo. The absence of these factors is apparently of greater consequence to type I than to type III when refolded in vitro.

However, we would caution that protease lability does not distinguish between early and late events in the folding mechanism, since completion of both would be required to confer resistance to the refolded structure. Alternatively, the use of CD for direct measurement of collagen helicity afforded the opportunity to observe early events in the refolding process. In particular, the 2-fold greater initial refolding rate observed for hupro(III) as compared to hucol(III) is thought to reflect the influence of the carboxyl propeptides. In view of studies cited above, which indicate that assembly intiates from the carboxyl end of procollagen, this influence may be of physiological significance.

In conclusion, we note that there have been few prior studies on the characterization of type III procollagen of human origin, presumably due to the difficulty in obtaining sufficient quantities for analysis. A human tumor cell line in culture has been reported recently which produces greater than 90% type III procollagen, as isolated from the culture medium (Krieg et al., 1979). As suggested in that report, this system should prove useful as a means for the continued study of the human type III collagen precursor.

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