Kinetics of Collagen Fold Formation in Human Type I Procollagen and the Effect of Disulfide Bonds†

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ABSTRACT: The postulated role of disulfide bonds in the kinetics of collagen fold formation has been investigated by circular dichroic studies of highly purified human type I procollagen. For these studies, procollagen was isolated from fibroblast culture medium by adsorption to controlled-pore glass beads and subsequently purified to relative homogeneity. Tadpole collagenase digestion of this material revealed the presence of both amino and carboxyl propeptides on both proα1 and proα2 chains. The amino acid composition reflects the contribution of these propeptides to that characteristic of type I collagen. The circular dichroic spectra for native and denatured procollagen were determined and found to be qualitatively similar to the corresponding spectra of soluble rat skin collagen, although quantitative differences were observed. These differences probably reflect in part a contribution by the propeptide structure. The rates of human type I procollagen renaturation were examined at 10, 20, and 30 °C following thermal denaturation at 45 °C. The rate of renaturation was found to be first order, concentration independent over a 3-fold concentration range, and directly related to the degree of undercooling. Reduction and alkylation of disulfide bonds, under conditions which left intact a major proportion of intrachain bonds in the carboxyl propeptides, strikingly decreased the rate of renaturation at each of the three temperatures examined, the effect becoming more marked at the higher temperatures. First-order rate constants obtained for renaturation at 20 °C were 38.1×10^{-5} s^{-1} for procollagen, 5.3×10^{-5} s^{-1} for reduced and alkylated procollagen, and 2.7×10^{-5} s^{-1} for rat skin collagen. The data support the hypothesis that disulfide bond formation in procollagen is an essential event in order that collagen fold formation proceed at a biologically appropriate rate.

Collagen is biosynthesized as a precursor containing noncollagenous extension peptides of considerable mass at both the amino and carboxyl termini (Fessler & Fessler, 1978; Prockop et al., 1979). A number of biosynthetic functions have been suggested for the procollagen propeptide sequences which include enhancement of solubility under physiological conditions (Schmitt, 1960), chain registration (Speakman, 1971), fibril formation (Veis & Brownell, 1975; Bruns et al., 1979), feedback regulation of collagen synthesis (Krieg et al., 1978; Wiestner et al., 1979), and facilitation of collagen fold formation (Speakman, 1971; Uitto & Prockop, 1973; Prockop et al., 1976). On the basis of an indirect enzymatic method of helix analysis, several recent reports suggest a possible relationship between interchain disulfide bonds in the carboxyl-terminal propeptides of proα[1] chains and collagen fold formation (Fessler et al., 1974; Schofield et al., 1974; Rosenbloom et al., 1976; Prockop et al., 1976; Harwood et al., 1977).

In this investigation, collagen fold formation in highly purified type I procollagen was directly monitored by circular dichroism (CD)[2] at several temperatures following thermal denaturation. This procedure allowed a comparison of the mutarotation rates for procollagen as a function of the integrity of disulfide bonds. Our results indicate that an optimal rate of triple helix formation in the collagen core of procollagen may require the prior formation of disulfide bonds either between chains, within the amino propeptides, or possibly at both loci.

Materials and Methods

Procollagen Isolation and Purification. Human type I procollagen produced by foreskin fibroblast cultures was isolated from the serum-free culture medium by adsorption to and differential elution from controlled-pore glass beads (Gerard & Mitchell, 1979). Procollagen was subsequently purified by DEAE-cellulose chromatography and finally by molecular sieve chromatography on Bio-Gel A-5m to yield the intact type I trimer, proγ. For certain experiments, proγ containing ^{3}H-labeled imino acids was isolated and purified, having included 1 μCi/mL L-[5-^{3}H]proline (Schwarz/Mann) in the culture medium during incubation. In all cases, the details of methodology were as previously described (Gerard & Mitchell, 1979).

NaDodSO$_4$–Polyacrylamide and NaDodSO$_4$–Agarose–Polyacrylamide Gel Electrophoresis. Electrophoresis of procollagen and collagen in the presence of NaDodSO$_4$ was performed on cylindrical gels which contained a composite of 2% acrylamide and 1% agarose. Tadpole collagenase digests of procollagen and collagen were electrophoresed on 5% acrylamide slab (0.15 × 11.0 cm) or cylindrical (0.6 × 10.5 cm) gels in the presence of NaDodSO$_4$. All electrophoretic procedures employed were otherwise as described previously (Gerard & Mitchell, 1979).

Amino Acid Analysis. Samples of hydrolyzed proγ were analyzed on a Beckman Model 120 amino acid analyzer. Performic acid oxidation by the method of Moore (1963) was used for determination of cysteine and methionine as cysteic acid and methionine sulfone, respectively. Cysteic acid re-

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[1] Definitions: proα, single collagen chain precursor with both amino- and carboxyl-terminal propeptides; proβ, two proα chains disulfide linked at the carboxyl propeptides; proγ, three proα chains disulfide linked at the carboxyl propeptides; pα, proα chain missing the carboxyl propeptide; A and B superscripts applied to collagen and procollagen forms are used to denote the amino and carboxyl fragments, respectively, of tadpole collagenase digestion.

[2] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; NaDodSO$_4$, sodium dodecyl sulfate; CD, circular dichroism; DTT, dithiothreitol; BSA, bovine serum albumin; BME, β-mercaptoethanol; ACS, aqueous counting scintillant; T_m, melting temperature.

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coveries were corrected by assuming a 94% recovery (Moore, 1963).

Protein Estimation. Proγ protein concentrations were estimated by tryptophan fluorescence using bovine serum albumin as standard as detailed previously (Gerard & Mitchell, 1979). Proγ hydrolyzates were also quantitated by using fluorescamine (Roche Diagnostics), a primary amine reagent (Bohlen et al., 1973), using as standard a mixture of amino acids based on the results of amino acid analysis. Comparison of quantitation by fluorescamine to estimation by tryptophan fluorescence of the procollagen solution prior to hydrolysis indicated that the tryptophan fluorescence value was low by a factor of 2.6. This discrepancy is in contrast to previous results (Gerard & Mitchell, 1979) in which similar estimates were obtained by tryptophan fluorescence and the method of Lowry et al. (1951). However, the accuracy of this correction factor was supported by agreement of quantitation based on amino acid analysis recoveries to within 10–15% with the estimates by primary amino assay. Tryptophan fluorescence estimates of proγ used for CD studies were therefore corrected by this factor.

Reduction and Alkylation. Proγ was reduced and alkylated under nondenaturing conditions by using a modification of the procedure of Dehm et al. (1974). As shown under Results, interchain disulfide bonds were almost completely cleaved under these conditions, but intrachain cleavage was partial and selective. Procollagen (4 mL at 120 μg/mL) was dialyzed at room temperature against 0.5 M Tris-HCl, pH 8.5, measured at 23 °C, containing 2 mM EDTA. Tris-HCl (0.1 M), pH 7.6, was used to make a 50 mM stock solution of dithiothreitol (DTT) and, immediately before use, a 133 mM stock solution of iodoacetamide which had been recrystallized from petroleum ether (Sherr et al., 1973). For experiments in which the extent of reduction was evaluated, iodo[1-^{14}C]acetamide (Amersham, 53 mCi/mmol) was substituted in the same molar ratio. DTT stock (40 μL) was added, the solution was gently vortexed and capped under nitrogen, and reduction was allowed to proceed for 40 min at room temperature. After addition of 40 μL of stock iodoacetamide, the incubation was continued in the dark at room temperature for 20 min. Reduced and alkylated proγ (RAproγ) was then dialyzed exhaustively in the dark at 4 °C against 0.5 M NaCl, Tris-HCl (50 mM), pH 8.2, measured at 4 °C, and 0.1 mM EDTA. Completeness of interchain reduction was examined by NaDodSO$_4$–polyacrylamide gel and NaDodSO$_4$–agarose–polyacrylamide gel electrophoresis.

Tadpole collagenase digested procollagen was reduced and alkylated under denaturing conditions as described above with the following exceptions. All buffers contained 1% NaDodSO$_4$, and iodo[^{14}C]acetamide was used for alkylation. Reduction was performed initially at 100 °C for 5 min and then continued at 37 °C for 35 min. Dialysis of reduced and alkylated procollagen in the dark was performed at room temperature.

Tadpole Collagenase Digestion. Crude tadpole collagenase was a generous gift from Dr. John Harper. Further purification of the crude enzyme was accomplished by affinity chromatography as described by Bauer et al. (1971), except that lathrytic rat skin collagen was used as the ligand. Digestions were performed at 23 °C in 0.5 M NaCl, 5 mM CaCl$_2$, and 50 mM Tris-HCl, pH 7.4, measured at 23 °C. Substrate concentrations in enzyme incubations were 150 μg/mL for proγ, 53 μg/mL for RAproγ, and 500 μg/mL for rat skin collagen. Enzyme concentration was estimated by tryptophan fluorescence, using BSA as standard, as described previously (Gerard & Mitchell, 1979). As such, enzyme:substrate ratios on a weight basis were 1:(1.3×10^4) for proγ, 1:(1.5×10^4) for RAproγ, and 1:(2.9×10^3) for rat skin collagen. In all cases, samples were incubated for 24 h, after which time a second aliquot of enzyme was added and incubation continued for an additional 24 h (Sage et al., 1979). Digestion was terminated by addition of $^1/_{20}$ volume of 20% NaDodSO$_4$ (Bio-Rad) and heating to 100 °C for 5 min.

Circular Dichroism. a. Sample Preparation. Purified proγ was dialyzed at 4 °C against 0.5 M NaCl, 50 mM Tris-HCl, pH 8.2, measured at 4 °C, and 0.1 mM EDTA and concentrated at 4 °C with dry Sephadex G-200. All procollagen used for CD was kept at 4 °C in the above buffer for at least 1 week subsequent to the final purification on Bio-Gel in 1 M CaCl$_2$, and CD spectra were taken of procollagen solutions in this buffer. For CD measurements on rat skin collagen, a weighed amount of lyophilized lathrytic rat skin collagen stored desiccated was solubilized in 1% acetic acid and then dialyzed at 4 °C against the above buffer described for procollagen.

b. Apparatus. Circular dichroic measurements were recorded on a Cary Model 60 spectropolarimeter equipped with a CD attachment using a jacketed quartz cell with a 5-mm pathlength. Temperature control between 10 and 50 °C was achieved by using two Lauda circulating water baths in turn connected to the cell jacket, calibrating the cell sample compartment temperature using a thermocouple.

For renaturation kinetics experiments, spectra were recorded first at an initial temperature of either 10, 20, or 30 °C, then at 45 °C, and finally over a subsequent time interval after quench cooling back to the initial temperature. Spectra at 45 °C were recorded about 5 min after the temperature shift, after which time no further change in θ_{223} was observed; all samples were maintained at this temperature for a total period of 20 min. Loss of sample due to precipitation, adsorption, or otherwise secondary to temperature manipulation was not apparent on the basis of visual inspection and tryptophan fluorescence of the sample at the end of the experiment.

The kinetics of the actual temperature shift inside the cell was determined for 45 to 10, 20, and 30 °C jumps by using a thermocouple. For each of the three jumps, the desired temperature was asymptotically approached inside the cell to within 0.5 °C after 2.5 min. The completeness of the temperature transition in percent of total shift for times between time zero and 2.5 min was similar for all three jumps: 50 after 0.5 min, 90 after 1.5 min, and 96 after 2 min.

c. Data Analysis. For the purposes of discussion, "native" and "denatured" will be used to refer to native collagen triple helix and random coil (i.e., heat denatured) collagen conformation, respectively, in the collagen sequences of both rat skin collagen and proγ. Spectra of collagen and proγ were normalized to specific mean residue ellipticity, $[\theta]$, which has conventional units of deg·cm^2/dmol, using eq 1, where θ is the

$$[\theta] = \theta(\mathrm{MRW})/(10lc) \quad (1)$$

corrected ellipticity in millidegrees, MRW is the mean residue weight, l is the path length in cm, and c in the concentration in mg/cm^3. A mean residue weight of 97.9 was calculated for human type I procollagen from the amino acid composition.

For kinetic analysis of collagen and proγ renaturation at 10 and 20 °C, it was assumed that the initial θ_{223} (i.e., prior to heat denaturation) corresponded to 100% collagen helix and that θ_{223} at 45 °C represented 0% collagen helix. Helix fraction, f, was calculated from the data at 223 nm by using eq 2, where θ_t is the ellipticity at time t at T (where T = 10,

$$f = (\theta_t - \theta_{45})/(\theta_T - \theta_{45}) \quad (2)$$

20, or 30 °C), θ_{45} is the ellipticity at 45 °C, and θ_T denotes

the ellipticity at T prior to heating.

The magnitude of the temperature-induced transition at 223 nm, $\Delta[\theta]_{223}$, was determined for thermal shifts from either 10, 20, or 30 °C to 45 °C by using eq 3. The mean value of

$$\Delta[\theta]_{223} = (\theta_T - \theta_{45})(\mathrm{MRW})/(10lc) \quad (3)$$

$\Delta[\theta]_{223}$ for seven conditions of procollagen at 10 and 20 °C equaled 4220 ± 70 deg·cm^2/dmol. The value associated with procollagen denaturation at 30 °C, however, was decreased in magnitude. Thus, the calculated values of f for the 30 °C procollagen renaturations were corrected by the factor $\Delta[\theta]^{30}_{223}/4220$, where $\Delta[\theta]^{30}_{223}$ is the respective 30–45 °C procollagen transition magnitude.

When applicable, first-order rate constants were determined over a specified time range by plotting $-\ln(1-f)$ against time in seconds and performing linear regression analysis on the data according to eq 4. In the case of the 45–30 °C renaturations, a curve which asymptotically approaches $-\ln[1 - (\Delta[\theta]^{30}_{223}/4200)]$ with equilibrium can be predicted for the semilog plots of the data as described by eq 4. The curvature of these plots over the time range analyzed, however, was sufficiently minimal so as to permit a linear fit of the data.

$$-\ln(1-f) = kt + \text{constant} \quad (4)$$

The pooled variance, $V(A/B)$, of the ratio of the first-order renaturation rates of proγ and its reduced and alkylated derivative were estimated by using a Taylor expansion series where

$$V(A/B) = (A/B)^2[V(A)/A^2 + V(B)/B^2] \quad (5)$$

in which A and B are first-order renaturation rate constants for proγ and for RAproγ, respectively, and $V(A)$ and $V(B)$ represent the variance of their respective rate constants. When the pooled variance as estimated by eq 5 is used, the critical ratio, Z, was obtained by eq 6 in order to evaluate the level

$$Z = [(A/B) - 1]/[V(A/B)]^{1/2} \quad (6)$$

of statistical significance of the ratio using a two-tailed t test.

Scintillation Counting. Radioactivity profiles of cylindrical 5% acrylamide gels were determined by slicing gels into 1-mm sections, solubilizing in 30% H_2O_2 at 85 °C, and then counting in 15 mL of ACS (Amersham) in a Beckman Model LS-233 liquid scintillation counter. Counting efficiencies were 18–22% for ^{3}H and 55–65% for ^{14}C.

Results

Demonstration of Propeptide Sequences in Human Type I Procollagen. Human type I proγ of high purity was obtained by adsorption to and elution from a controlled-pore glass bead column, subsequent resolution of genetic types on DEAE-cellulose, and final purification by molecular sieve chromatography on Bio-Gel A-5m, as previously described (Gerard & Mitchell, 1979). The relative homogeneity of this material is demonstrated in Figure 1a, gel 1. Note that the electrophoretic mobility of the trimeric disulfide cross-linked collagen precursor in NaDodSO$_4$–agarose–polyacrylamide gel electrophoresis is considerably retarded as compared to the aldehyde cross-linked γ collagen form of rat skin collagen (Figure 1a, gel 5). As described previously, reduction of proγ (Figure 1a, gel 2) yields its component proα1 and proα2 bands in the expected 2:1 ratio, respectively, with retarded electrophoretic mobilities relative to the α1 and α2 bands of rat skin collagen.

Figure 2 shows the Coomassie blue stained electrophoretic profiles on 5% NaDodSO$_4$–polyacrylamide gel electrophoresis of tadpole collagenase digested proγ and collagen and of procollagen and collagen standards. Track 1 of Figure 2, which represents material from the 1 M Tris-HCl eluate from glass beads, displays the various procollagen forms, including proγ, proβ, and pα, as were previously described and resolved by NaDodSO$_4$–agarose–polyacrylamide gel electrophoresis (Gerard & Mitchell, 1979). Note that in 5% NaDodSO$_4$–polyacrylamide gel electrophoresis, pα1 (Figure 2, track 1)

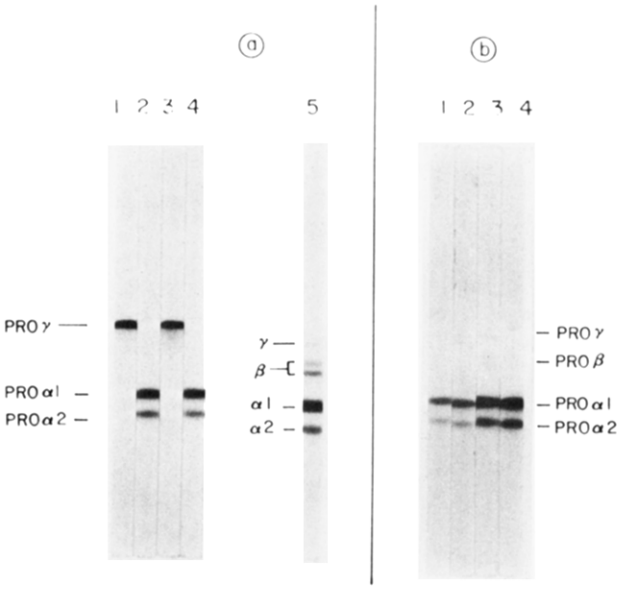

FIGURE 1: NaDodSO$_4$–agarose–polyacrylamide gel electrophoresis of proγ and reduced and alkylated proγ. Migration is from top to bottom. (a) gel 1, proγ; gel 2, proγ reduced with 1% β-mercaptoethanol; gel 3, proγ which had been heated to 45 °C for 20 min and then kept at 20 °C for 5000 s for renaturation studies; gel 4, same as gel 3 reduced with 1% β-mercaptoethanol; gel 5, rat skin collagen. (b) gel 1, reduced and alkylated proγ; gel 2, reduced and alkylated proγ reduced with 1% β-mercaptoethanol; gel 3, reduced and alkylated proγ which had been heated to 45 °C for 20 min and then kept at 20 °C for 5000 s for renaturation studies; gel 4, same as gel 3 reduced with 1% β-mercaptoethanol.

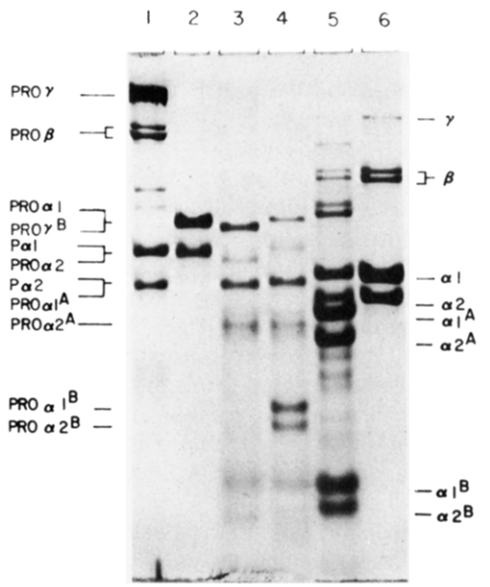

FIGURE 2: NaDodSO$_4$–polyacrylamide gel electrophoresis of procollagen, collagen, and tadpole collagenase digests. samples were electrophoresced on a 5% slab gel. Migration is from top to bottom. Track 1, partially purified procollagen of the 1 M Tris-HCl glass bead eluate; track 2, purified proγ reduced and alkylated under nondenaturing conditions; track 3, tadpole collagenase digest of proγ; track 4, tadpole collagenase digest of proγ, reduced and alkylated under denaturing conditions; track 5, tadpole collagenase digest of rat skin collagen; track 6, rat skin collagen.

and proα2 (Figure 2, track 2) are observed to comigrate, in contrast to their relative mobilities in $NaDodSO_4$–agarose–polyacrylamide gel electrophoresis (Gerard & Mitchell, 1979).

Tadpole collagenase digested proγ is shown in tracks 3 and 4 of Figure 2 electrophoresed unreduced and reduced and alkylated, respectively. The band in track 3 with a mobility slightly greater than proα1 (track 2) is identified as proγ^B, the disulfide-linked carboxyl fragment trimer. Upon reduction, this band is replaced by its more rapidly migrating pro$\alpha 1^B$ and pro$\alpha 2^B$ components (Figure 2, track 4). The mobilities of these proα^β fragments are considerably retarded with respect to their corresponding $\alpha 1^B$ and $\alpha 2^B$ bands of the collagen digest (Figure 2, track 5). A small amount of material seen under reducing conditions in the position of proγ^B (Figure 2, track 4) represents proα1, derived from reduction of undigested proγ (Figure 2, track 3).

The pro$\alpha 1^A$ fragment is represented by a prominent band under nonreducing or reducing conditions (Figure 2, tracks 3 and 4) migrating just ahead of the α1 band of collagen (Figure 2, tracks 5 and 6). The mobility of this band is clearly retarded with respect to the $\alpha 1^A$ band of collagen (Figure 2, track 5). Three closely spaced bands, of which the center band is most prominent, are seen in tracks 3 and 4 of Figure 2 at approximately the level of the α^A bands of the collagen digest (Figure 2, track 5). Since the slowest migrating band of these three is seen to comigrate with the $\alpha 1^A$ band of track 5, it is believed that this band represents some $\alpha 1^A$ generated during the incubation. The center and most prominent band of the triplet is believed to represent the pro$\alpha 2^A$ fragment, with a retarded mobility as compared to the $\alpha 2^A$ band (Figure 2, track 5). The identity of the fastest migrating band of these three is uncertain but may represent a truncated form of the pro$\alpha 2^A$ fragment, presumably generated by contaminating proteolytic activity.

Several rapidly migrating diffuse bands are seen in the lower region of tracks 3 and 4 of Figure 2. These bands most likely represent α^B fragments and some free propeptides generated during the enzyme incubation. The protease(s) responsible for these modifications as well as for some of the others discussed above may have copurified by affinity chromatography, since small quantities of rat procollagen, as visualized by 5% $NaDodSO_4$–polyacrylamide gel electrophoresis (not shown), are present in the lathrytic rat skin extracts used as ligand. Alternatively, small amounts of protease may contaminate the proγ preparations. It is believed that the prolonged incubation period under favorable conditions, i.e., 48 h at 23 °C in 5 mM $CaCl_2$, permitted expression of this contaminating activity. Nevertheless, the proα^A and proα^B tadpole collagenase fragments identified for both proα1 and proα2 chains demonstrate the existence of propeptide sequences at the amino and carboxy termini in proγ isolated and purified by the described methodology. Additionally, these results establish the presence of interchain disulfide bonds in the carboxyl fragments but not in the amino fragments of proγ.

Amino Acid Analysis. Apparent similarities in the composition of procollagen and human skin collagen (Table I) are reflective of the collagen molecule contained within procollagen. These similarities include the presence of hydroxyproline and hydroxylysine and the significant content of glycine, total imino acids, and alanine. Differences between the composition of procollagen and human skin collagen include the presence in the former of cysteine, increased content of acidic residues, tyrosine, and phenylalanine, and decreased proportion of glycine, alanine, and imino acids. The presence of tryptophan in procollagen, which is absent in collagen, was demonstrated by tryptophan fluorescence but was not quantitated in this analysis.

Table I: Amino Acid Composition of Human Type I Procollagen[a]

residue	human type I procollagen	human skin collagen[b]
4-Hyp	74	91
Asp	64	47
Thr	27	18
Ser[c]	41	37
Glu	85	78
Pro	108	125
Gly	262	324
Ala	97	115
$^1/_2$-Cys[d]	12	
Val	34	25
Met[e]	7.9	7.1
Ile	19	10
Leu	34	25
Tyr	13	3.5
Phe[c]	22	13
Hyl	7.1	5.9
His	7.9	5.4
Lys	35	27
Arg	50	49
Trp	ND[f]	

[a] Values are expressed as residues/1000. Results for procollagen are a composite of nine separate analyses. [b] As taken from Fleischmajer & Fishman (1965). [c] Corrected for hydrolytic loss. [d] Determined as cysteic acid. [e] Determined as methionine sulfone. [f] Not determined.

Characterization of Partially Reduced and Alkylated Procollagen. The reduction/alkylation procedure as utilized herein proved to be effective in reducing the interchain disulfide bonds in proγ, as evidenced by $NaDodSO_4$–agarose–polyacrylamide gel electrophoresis shown in Figure 1b. RAproγ is shown in gel 1 and gel 2 electrophoresed in the absence and presence of 1% BME, respectively. Only a relatively small amount of proγ (trimer) and proβ (dimer) forms are seen in gel 1. Gels 3 and 4 of Figure 1b show denatured/renatured RAproγ electrophoresed with and without 1% BME, respectively. Since about twice the amount of sample was applied to these two gels as compared to gels 1 and 2, gel 3 illustrates more clearly the small amount of unreduced proγ and proβ forms. Radioactivity profiles of ^{3}H-labeled RAproγ electrophoresed on 5% $NaDodSO_4$–polyacrylamide gel electrophoresis (not shown) revealed approximately 10% of the total material migrating as proγ and proβ forms. Since virtually all of the radioactivity was distributed as either proγ, proβ, or proα bands, a 90% yield of reduction of interchain disulfide bonds can be estimated under the conditions as described.

For investigation of the extent of reduction under nondenaturing conditions of total disulfide bonds in amino and carboxyl propeptides, ^{3}H-labeled proγ was reduced and alkylated under these conditions using iodo[^{14}C]acetamide and then digested with tadpole collagenase. Additionally, ^{3}H-labeled proγ, first digest with tadpole collagenase, was reduced and alkylated under denaturing conditions (i.e., 1% $NaDodSO_4$) also using iodo[^{14}C]acetamide. The fragments of the digests were resolved by 5% $NaDodSO_4$–polyacrylamide gel electrophoresis (not shown), and the ratios of iodo[^{14}C]acetamide to ^{3}H-labeled imino acids were determined for the respective gel bands as shown in Table II. These results show that, under nondenaturing conditions, a major proportion of the amino propeptide disulfide bonds in human type I procollagen are reduced but that only a smaller fraction of total disulfide bonds in the carboxyl propeptides have been reduced. The results summarized in Table II taken in conjunction with

Table II: Extent of Total Disulfide Bond Reduction in Amino and Carboxyl Propeptides of Tadpole Collagenase Digested Procollagen[a]

gel band	iodo[^{14}C]acetamide/^{3}H-labeled imino acids[b] nondenaturing reduction	denaturing reduction	nondenaturing/denaturing (%)
proα1^{A}	1.32	2.08	63
proα2^{A}	0.42	0.52	81
proα1^{B}	0.83	2.92	28
proα2^{B}	0.85	2.53	34

[a] ^{3}H-Labeled proγ (^{3}H-labeled imino acids) was reduced and alkylated, using iodo[^{14}C]acetamide, either prior to or following tadpole collagenase digestion under nondenaturing or denaturing conditions of reduction, respectively. Amino and carboxyl fragments of the collagenase digests were resolved by 5% NaDodSO$_4$-polyacrylamide gel electrophoresis. [b] Radioactivity ratios are based on dpm values of the respective gel bands.

Table III: Circular Dichroic Values of Procollagen and Collagen

sample	μg/mL	$[\theta]_{223}$[a] 20 °C	45 °C	$\Delta[\theta]_{223}$[a]
human type I procollagen	72	2400	−1800	4200
rat skin collagen	100	6350	−1250	7600

[a] Units are deg·cm^2/dmol.

the NaDodSO$_4$-electrophoretic profiles of RAproγ (Figure 1b) are consistent with the interpretation that nondenaturing conditions allow for successful reduction of interchain bonds but of only a minor proportion, if any, of the intrachain bonds in the carboxyl propeptides of human type I procollagen.

Conformational Analysis of the Collagen Fold. The far-ultraviolet CD spectrum of collagen is characterized by a positive peak at 223 nm and a larger negative extremum at 197 nm (Tiffany & Krimm, 1969). The 223-nm band was used to monitor the presence of the collagen fold because of the increased accuracy afforded by a better signal to noise ratio at this wavelength as well as the clear distinction between the positive extremum at 223 nm characteristic of the collagen helix and the negative contributions of aperiodic coil at this wavelength (Tiffany & Krimm, 1969).

The CD spectra of native and denatured type I proγ and lathrytic rat skin collagen are illustrated in Figure 3 with corresponding values shown in Table III. Both native procollagen and collagen at 20 °C show the characteristic positive CD band at 223 nm. A specific ellipticity for rat skin collagen of 6350 deg·cm^2/dmol was observed, which is in good agreement with previous reports for this collagen (Piez & Sherman, 1970; Bankowski & Mitchell, 1973; Brodsky-Doyle et al., 1976). The positive peak at 223 nm for proγ was observed to be less positive with a specific ellipticity of 2400 deg·cm^2/dmol.

The CD spectra for denatured rat skin collagen and human proγ, taken at 45 °C, are similar in that both have undergone a transition from a positive peak at 223 nm to a negative value (Figure 3). However, the magnitude of the negative value for procollagen, $[\theta]_{223} = -1800$ deg·cm^2/dmol, is greater than for rat skin collagen, $[\theta]_{223} = -1250$ deg·cm^2/dmol. Thus, the CD spectra observed for both native and denatured proγ are shifted in a negative direction as compared to their respective rat skin collagen CD spectra.

Essentially the identical CD spectra as shown in Figure 3 for native proγ at 20 °C and for heat-denatured proγ at 45 °C were obtained for RAproγ under these two conditions. Significantly, therefore, reduction and alkylation of procollagen

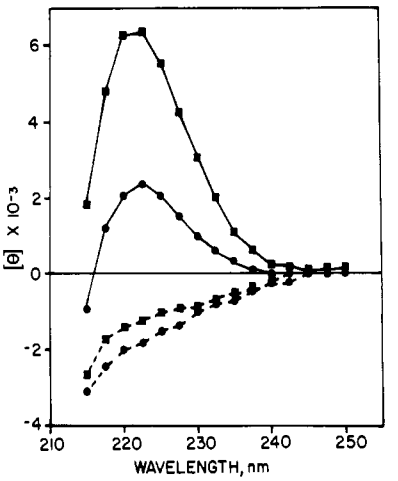

FIGURE 3: Circular dichroic spectra of native and denatured collagen and procollagen. Circular dichroic spectra of native forms, measured at 20 °C (solid line), and of denatured forms, measured at 45 °C (broken line), are shown for lathrytic rat skin collagen (■) and human type I procollagen (●). Concentrations were 100 μg/mL for collagen and 72 μg/mL for procollagen.

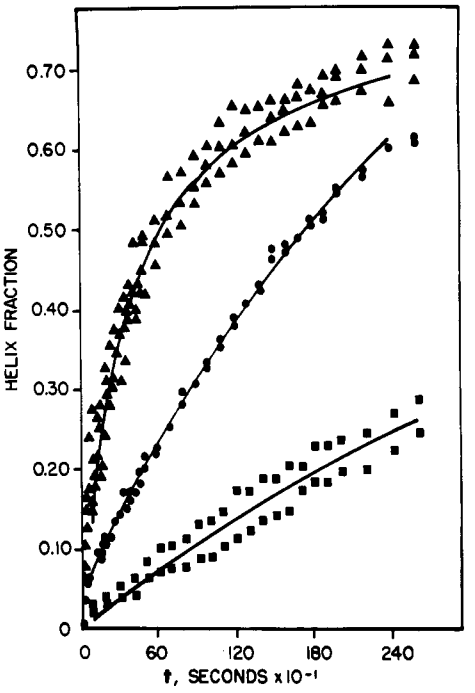

FIGURE 4: Procollagen renaturation at 10, 20, and 30 °C. Human type I procollagen renaturation is shown at 10 (▲), 20 (●), and 30 °C (■). Concentrations of procollagen were 72 and 107 μg/mL for 10 °C, 215 μg/mL for 20 °C, and 72 μg/mL for 30 °C.

under the conditions used did not detectably alter the extent of the collagen helix in procollagen.

Heat Stability of Procollagen. Electrophoresis of proγ subsequent to heat denaturation/renaturation as employed in the CD protocol for kinetic analysis of renaturation rates is shown in Figure 1A. Comparison of electrophoretic profiles of identical amounts of sample, normalized by tryptophan fluorescence, before and after temperature manipulation in the CD cell reveals little difference. The nearly identical NaDodSO$_4$–agarose–polyacrylamide gel electrophoresis band intensities of unreduced (gels 1 and 3) or reduced (gels 2 and 4) samples indicate no significant alteration of proγ under these conditions.

Kinetics of Collagen Fold Re-formation. Collagen fold re-formation by heat-denatured proγ at 10, 20, and 30 °C is illustrated in Figure 4. At 20 °C, the mutarotation rate could be resolved into three phases. During the first 60–70 s, there

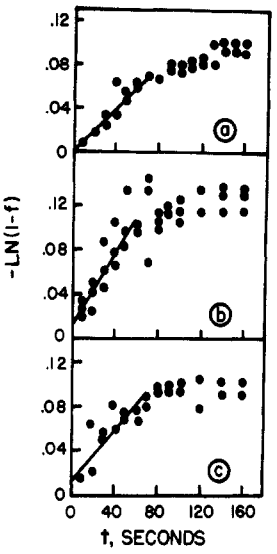

FIGURE 5: Early rapid phase of procollagen and collagen renaturation at 20 °C. Each sample was heated to 45 °C for 20 min and then cooled to 20 °C and ellipticity recorded. (a) procollagen at 215 μg/mL (two samples); (b) reduced and alkylated procollagen at 110 μg/mL (two samples) and 165 μg/mL (1 sample); (c) rat skin collagen at 100 μg/mL (two samples).

was a rapid apparent first-order rate for which $k \simeq 10^{-3}$ s^{-1}. Figure 5 illustrates this initial phase of collagen fold re-formation at 20 °C for proγ, RAproγ, and rat skin collagen. The apparent rate constant of this initial phase for proγ (Figure 5a) is equal to that of collagen (Figure 5c) and appears to be slightly less than that of RAproγ (Figure 5b) for which $k \simeq 1.6 \times 10^{-3}$ s^{-1}.

The major phase of CD change, which follows the initial rapid phase at 20 °C, was a highly temperature-dependent process (Figure 4). The time interval of the first-order character of the rate was observed to depend on the temperature of renaturation (Figure 6). At 10 °C, the rate of collagen fold reformation was first order only to about 500 s (Figure 6a). The rate at 20 °C (Figure 6b) was approximately 2.7 times slower than the 10 °C rate (Table IV), and the semilog plot was linear to 2400 s. Identical rate constants observed at 72 and 215 μg/mL (Table IV) demonstrate that

Table IV: First-Order Rate Constants of Renaturation

T (°C)	condition (N)[a]	μg/mL	time range[b]	10^5k (SD)[c]	$\Delta[\theta]_{223}$[d]
10	proγ (2)	72	100–500	101.0 (9.0)	4150[e]
10	proγ (1)	107	100–500	101.0 (3.0)	4250[e]
10	RAproγ (2)	72	100–500	40.0 (3.0)	4240[e]
20	proγ (5)	72	120–1500	38.1 (1.0)	4300[e]
20	proγ (2)	215	120–1500	37.8 (0.4)	4890
20	RAproγ (2)	72	100–1000	5.3 (0.9)	4110[e]
20	RAproγ (2)	110	200–1000	5.6 (0.9)	4290[e]
20	RAproγ (1)	165	100–1000	7.6 (0.4)	4210[e]
20	RSC (2)	100	100–1000	2.7 (0.6)	7600
30	proγ (2)	72	200–2000	11.6 (0.7)	3180
30	RAproγ (2)	72	200–2000	0.2 (0.4)[f]	2470

[a] Proγ, unreduced procollagen; RAproγ, reduced and alkylated procollagen; RSC, rat skin collagen; N, number of replicate samples. [b] In seconds. [c] In seconds^{-1}. [d] Units are deg·cm^2/dmol. [e] For these values, the mean = 4220 ± 70. [f] Positive value not significantly different from zero by the F test.

the process is concentration independent over this range. At 30 °C, the semilog plot could be fit by linear regression to at least 2400 s (Figure 6c) and was 3.3 times slower, as shown in Table IV, than collagen fold renaturation at 20 °C.

A considerably slower third phase predominates at approximately 2600 s for renaturation at 20 °C (data not shown). However, due to the slow rate of renaturation and considerable scatter of the data beyond 2400 s at each of the three temperatures, kinetic analysis of renaturation at times greater than 2400 s was not considered further.

In addition to proγ collagen fold reformation, Figure 6 illustrates the effect of partial reduction and alkylation of proγ on the renaturation rates at 10, 20, and 30 °C. In each of the illustrated comparisons, procollagen concentration was 72 μg/mL, with the exception of one 10 °C proγ sample at 107 μg/mL. These data as well as rates observed at other protein concentrations are summarized in Table IV. At each temperature, reduction and alkylation results in a significant retardation of collagen fold re-formation. As was the case for proγ, the linearity of the semilog plot for RAproγ is apparent only for 500 s at 10 °C (Figure 6a). At 20 °C, the semilog plot for RAproγ is linear only up to 1000 s, less than one-half that observed for proγ (Figure 6b). A plot for rat skin collagen

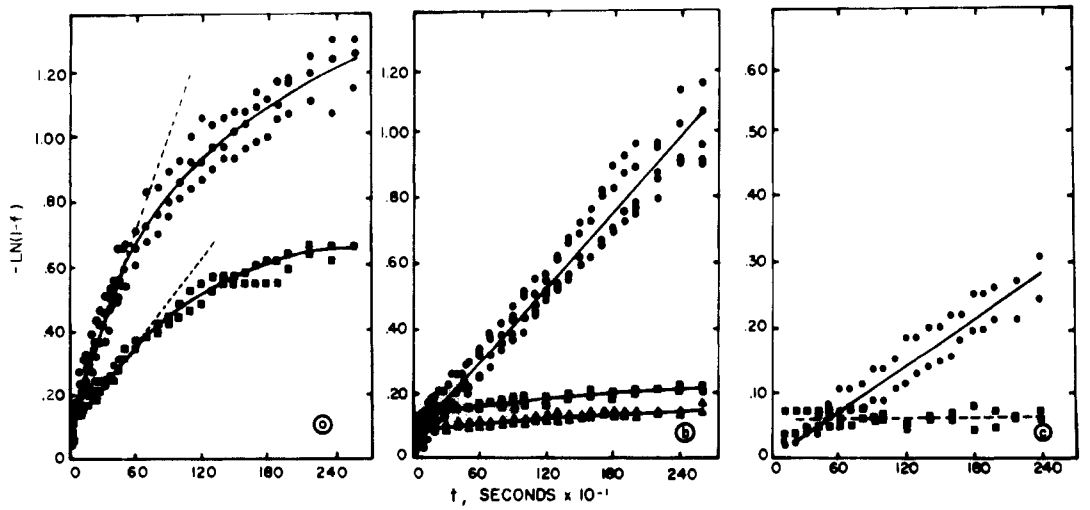

FIGURE 6: Major renaturation phase of procollagen and reduced and alkylated procollagen. Semilog plots are shown for procollagen (●), reduced and alkylated procollagen (■), and rat skin collagen (▲). (a) Renaturation at 10 °C with procollagen at 72 μg/mL (two samples) and 107 μg/mL (one sample) and reduced and alkylated procollagen at 72 μg/mL (two samples). The plots shown are linear up to 500 s (extended with a broken line) and then were continued by a visually estimated best fit smooth curve. (b) Renaturation at 20 °C with procollagen (five samples), reduced and alkylated procollagen (two samples) at 72 μg/mL, and rat skin collagen (two samples) at 100 μg/mL. (c) Renaturation at 30 °C with procollagen (two samples) and reduced and alkylated procollagen (two samples) at 72 μg/mL. The broken line drawn for the reduced and alkylated procollagen data represents a linear regression of the data plotted from 200 to 2000 s but does not reflect a positive slope of statistical significance, as indicated by the F test.

at 100 μg/mL concentration is included for comparison and is characterized by a first-order rate constant one-half that of RAproγ (Table IV).

When the rates observed for the major phase of renaturation are used, the (proγ rate/RAproγ rate) ratios are seen to be directly related to the temperature of refolding. At 10 and 20 °C, the ratios calculated are 2.5 and 7.2, respectively. At 30 °C, a positive slope for the RAproγ semilog plot over any time period after 100 s is not statistically significant, as indicated by the F test (Figure 6c, Table IV), due to the shallowness of the slope and the scatter of the data. However, with $p < 0.05$, the ratio of proγ and RAproγ renaturation rates was estimated to be >12 by using a two-tailed t test (see Materials and Methods). Thus, as the temperature of renaturation increases and approaches the T_m, the difference factor between proγ and RAproγ renaturation rates becomes increasingly larger.

Renaturation at 20 °C of two higher concentrations of RAproγ was examined. The highest concentration investigated was 165 μg/mL, which showed a first-order rate constant about 44% larger than for the 72 μg/mL renaturation (Table IV). This difference is suggestive of a concentration dependence of the rate of renaturation of RAproγ. However, insufficient quantities of RAproγ prohibited investigation of renaturation of higher concentrations to further substantiate a concentration dependence of the rate constant.

Discussion

In this investigation, we have attempted to further pursue the role of disulfide bonds in collagen fold formation in procollagen. That being our goal, we felt it necessary to characterize the propeptide and amino acid composition of human type I procollagen isolated by adsorption to controlled-pore glass beads, as was used in these studies. In regard to the former, our results do indeed demonstrate the presence of precursor sequences at both the amino and carboxy termini of proα1 and proα2 chains of procollagen isolated by our methodology and in so doing further establish the validity of our isolation/purification scheme. Similar results for the $NaDodSO_4$ electrophoretic profiles of animal collagenase-digested human fibroblast procollagen were previously reported (Lichtenstein et al., 1976), including a marked tendency for precipitation of the carboxyl fragments generated during the digestion. However, the electrophoretic profiles of the various collagenase fragments shown in Figure 2 herein represent a significant improvement in resolution and may be related in part to a lack of any such precipitation noted during our enzyme incubations. In the $NaDodSO_4$ electrophoretograms of tadpole collagenase-digested proγ (Figure 2, tracks 3 and 4), the assumption was made that the most predominant band of the triplet located in the region of the α^A bands represents $pro\alpha2^A$. If this assumption is correct, then a greater increment of retarded mobility is observed between the $pro\alpha1^A$ and $\alpha1^A$ bands than for the $pro\alpha2^A$ and $\alpha2^A$ bands. This observation is consistent with a proα2 amino-terminal propeptide of lesser mass as compared to the proα1 counterpart in human type I procollagen, as has been previously reported for other species (Prockop et al., 1979).

With respect to amino acid composition, differences between human type I procollagen and human skin collagen (Table I) reflect the contribution by the propeptide sequences in procollagen and are consistent with previous reports of amino acid composition for the human (Sherr et al., 1973), chick tendon (Olsen et al., 1977), and chick bone (Murphy et al., 1975) carboxy-terminal propeptide and for the sheep amino-terminal propeptide (Becker et al., 1976). Comparison of the amino acid composition determined in this investigation to that reported by Church & Tanzer (1975) for human type I procollagen, also isolated from fibroblast culture medium, reveals qualitative similarities but also some quantitative differences. Type I procollagen purified by the described methodology contains 2.5 times as much cysteine, 10% more aspartic acid, 6% more glutamic acid, 6% less glycine, 5% less alanine, and about one-third more isoleucine than was reported by Church and Tanzer. These differences were in each case at least two times greater than the range specified by the 95% confidence limits determined for each residue using the t statistic on the raw data. Each of these differences represents a greater deviation from the composition of human skin collagen than is indicated by the results of Church and Tanzer. Since $NaDodSO_4$–agarose–polyacrylamide gel electrophoresis profiles of proγ as purified herein (Figure 1a, gels 1 and 2) attest to its relative purity, it is believed that these differences may reflect the composition of a more highly purified procollagen than that analyzed by Church and Tanzer.

The observed CD spectra of procollagen taken at native and denaturing temperatures were similar to but negatively displaced as compared to their respective collagen spectra. Qualitatively similar CD spectral differences have recently been reported for chick and human type I procollagens (Hayashi et al., 1979; Peltonen et al., 1980). However, the procollagen CD spectral parameters described in both of those reports are of questionable validity since polyacrylamide autoradiograms of reduced procollagen samples reveal substantial amounts of contaminating material migrating with the proα2 band.

When a molecular weight for procollagen of 435 000 (Bornstein & Traub, 1979; Sandell & Veis, 1980) is assumed, of which one-third is propeptide sequences, some estimation of the propeptide secondary structure can be made based on the observed collagen and procollagen CD values shown in Table III. Adjusting the 20 and 45 °C collagen CD data by the factor 285/435 (i.e., the ratio of collagen and procollagen molecular weights) yields predicted values for the corresponding procollagen spectra if the propeptide sequences were to make no contribution. The observed specific ellipticity at 223 nm for procollagen at 45 °C is thereby found to be approximately 1000 deg·cm²/dmol more negative than the value predicted from the collagen CD spectrum. A somewhat greater negative shift of 1760 deg·cm²/dmol is obtained when comparing the 20 °C CD parameters and is believed to reflect both a lack of completely helical collagen structure in procollagen prior to heating (see below) and the negative spectral shift described above for the 45 °C condition. We conclude that these differences reflect a contribution by the propeptide structure of −1000 deg·cm²/dmol to the CD spectra at 223 nm of human type I procollagen. In view of the one-third mass fraction of total propeptides with respect to procollagen, a specific mean residue ellipticity of −3000 deg·cm²/dmol can be attributed to these sequences. This analysis thereby places an upper limit of 10% for the content of α helical conformation in the total propeptide sequences, based on the CD extrema of approximately −30 000 deg·cm²/dmol for this secondary structure (Chen et al., 1974). This estimate is compatible with conformation studies on the sheep proα1 amino-terminal propeptide (Engel et al., 1977).

It should be recalled that Bio-Gel chromatography in 1 M $CaCl_2$ was used for final purification of proγ. Since this represents a denaturing environment with respect to the helical confirmation, the collagen helicity observed in proγ depends on the extent to which the folded structure had re-formed at

4 °C in 0.5 M NaCl, 0.1 mM EDTA, and 50 mM Tris-HCl, pH 8.2, during the 1 week or more since the final purification step. Two considerations suggest that under these conditions helix re-formation in procollagen was not complete. First, when the mass ratio of 285/435 described above and the CD transition magnitude observed for rat skin collagen (Table III) were used, a $\Delta[\theta]_{223}$ of 4980 deg·cm^2/dmol would be predicted for procollagen. The observed $\Delta[\theta]_{223}$ for procollagen was 4200 deg·cm^2/dmol, or about 84% of the predicted value, indicating about a 16% lack of renatured helical structure. Second, the proγ melting curve for the temperature-induced helix-to-coil transition (not shown) revealed an apparent T_m, as defined by the temperature at which 50% of the transition had occurred, of 34.5 °C with a ΔT_t of 5 °C, where ΔT_t represents the width of the middle 50% of the transition (Hauschka & Harrington, 1970a). The low T_m and broadness of the transition also indicate an absence of complete helicity, as previously characterized for cross-linked icthyocol (Hauschka & Harrington, 1970b).

However, since the magnitude of $\Delta[\theta]_{223}$ for the procollagen 20–45 °C shift remained constant even after a 3-month period of incubation at 4 °C (data not shown), we conclude that the 84% helicity as estimated above can be assumed to represent an equilibrium value for the sample prepared under the described conditions. As such, the observed $\Delta[\theta]_{223}$ for procollagen of 4200 deg·cm^2/dmol was assumed to represent a relative value of 100% helicity for the purposes of kinetic analysis of procollagen renaturation.

The first-order rate behavior of collagen fold formation and rate independence of total protein concentration previously described for cross-linked collagens (Altgelt et al., 1961; Drake & Veis, 1964; McBride & Harrington, 1967) was also observed in this investigation for intact disulfide-linked human type I procollagen. Likewise, the rate of procollagen renaturation was directly related to the degree of undercooling, as previously described for rat tendon collagen (Flory & Weaver, 1960). An initial rapid phase of renaturation during the first 60–70 s was observed for both procollagen and collagen which accounted for approximately 8% of the total initial helicity. An initial rapid phase has been previously observed in the refolding of other collagen-containing proteins but has not been kinetically resolved (Engel et al., 1977; Brukner et al., 1978). From the pooled amino acid sequence data of calf and rat (Piez, 1976; Fietzek & Kühn, 1976), 8% of the α1 chain residues is accounted for by contiguous pyrrolidines (i.e., -Gly-Pro-(H)Pro-). It is therefore possible that the observed rapid phase represents a nucleation of these contiguous imino acids dispersed throughout the length of the α chain, which are particularly disposed to form the polyproline II configuration in the formation of the collagen fold (Harrington & von Hippel, 1961; Josse & Harrington, 1964).

For comparison of renaturation kinetics, reduction and alkylation of procollagen were performed under nondenaturing conditions. Incorporation of iodo[^{14}C]acetamide into tadpole collagenase fragments (Table II) allowed us to estimate the extent of reduction in both amino and carboxyl propeptides of RAproγ used in these studies. Extent of reduction of total disulfide bonds under nondenaturing conditions determined by direct measurement of carboxymethylcysteine was previously reported to be complete for the sheep type I amino-terminal propeptide (Engel et al., 1977) and approximately 50% for the chick type I carboxyl propeptide (Olsen et al., 1977). These values differ somewhat from the percent nondenaturing/denaturing ratios listed in Table II. Interspecies variation of propeptide sequences and differences in the lability of disulfide bonds in the intact procollagen molecule and the isolated propeptides might allow for some variation in extent of reduction under these conditions. Alternatively, nonspecific incorporation of iodo[^{14}C]acetamide may have introduced some error in the percent nondenaturing/denaturing ratios listed in Table II. We hesitate therefore to extrapolate directly from these ratios to percent of disulfide bond reduction in RAproγ propeptides. Nontheless, we believe that the data listed in Table II and the $NaDodSO_4$ electrophoretic analyses of ^{3}H-labeled proγ do justify a conclusion that almost all of the interchain disulfide bonds, as well as most of the intrachain disulfide bonds in the amino-terminal propeptides in RAproγ, were reduced, but that the major proportion of intrachain bonds in the carboxyl propeptides remained intact. This interpretation is consistent with the previous characterizations of the isolated propeptides (Engel et al., 1977; Olsen et al., 1977).

A mechanism for association of proα chains was originally postulated by Speakman (1971). The hypothesis proposed that additional precursor sequences, either at the amino or carboxy termini, were responsible for mediating chain registration of proper specificity and would subsequently facilitate helix formation. Subsequent studies with cultured chick bone (Fessler & Fessler, 1974) and chick tendon cells (Harwood et al., 1977) have shown that under conditions where helix formation is inhibited, type I proα chains have the ability to associate with the proper specificity and to become disulfide linked at the carboxyl propeptides. This implies that interchain association of the carboxyl propeptides does not depend on prior helix formation and, therefore, may precede it in accordance with the Speakman hypothesis.

The facilitation of helix formation by the carboxy-terminal interchain disulfide bonds has been difficult to demonstrate experimentally. The most commonly used technique has been the correlation of disulfide bonding to helicity analyzed as a function of resistance to proteolysis by pepsin or chymotrypsin (Fessler et al., 1974; Schofield et al., 1974; Rosenbloom et al., 1976; Prockop et al., 1976; Harwood et al., 1977). However, since incubations with these proteases were commonly performed at reduced temperature for extended periods of time, it is not known to what extent protease resistance correlates to collagen helicity at the time of isolation (Kao et al., 1977; Fessler & Fessler, 1978). Other studies using optical rotatory dispersion analysis have demonstrated that heterogeneous preparations of collagen precursors more rapidly form the collagen fold than does collagen (Veis et al., 1973; Byers et al., 1974). However, no evidence was obtained to correlate this increased rate to the presence of disulfide bonds.

The studies described in our investigation provide an alternative approach to this problem in that helicity as a function of disulfide bonds was directly determined by CD analysis. Interpretation of our data in conjunction with previous collagen folding studies supports a conclusion that the marked differences between the renaturation rates of proγ and RAproγ are attributable to the carboxyl interchain disulfide bonds. First, the relationship between the renaturation rates of procollagen and its reduced and alkylated counterpart is very similar to the differences between the refolding rates of previously studied cross-linked and free-chain collagens (Altgelt et al., 1961; Drake & Veis, 1964; McBride & Harrington, 1967). Specifically, the direct temperature dependence of the difference factor between proγ and RAproγ renaturation rates is highly analogous to the same type of relationship described for cross-linked and free-chain icthyocol (Hauschka & Harrington, 1970). In addition, reduction and alkylation of the

cyanogen bromide liberated sheep pα1 amino-terminal propeptide did not result in any significant difference in the rate of helix formation in the collagen-like portions of the propeptide (Engel et al., 1977). However, the behavior of the isolated amino propeptide in this instance may or may not be representative of these amino-terminal sequences in the intact procollagen molecule.

In the final analysis, it should be appreciated that our data do not discriminate between the relative contributions of the carboxy-terminal interchain and the amino-terminal intrachain disulfide bond populations. Nonetheless, our results clearly demonstrate the importance of disulfide bonds in the process of collagen fold formation in the intact procollagen molecule, consistent with those studies cited earlier using less direct methods of helix analysis. Furthermore, we have described herein an experimental system which we believe to be ideally suited for further investigation of this problem as well as for the study of potential biological mediators of helix formation in procollagen.

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