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Monomer and Oligomer of Type I Collagen: Molecular Properties and Fibril Assembly

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ABSTRACT: Type I collagen purified from calf skin was further separated into monomeric and oligomeric fractions and characterized with gel electrophoresis and measurement of solution viscosity. The thermal stabilities of the triple-helical structure of the collagen molecules of these preparations and the fibrils assembled therefrom were determined with differential UV spectroscopy and scanning microcalorimetry. The monomeric collagen was reduced with $NaBH_4$, and the kinetics and equilibrium of the reversible fibril assembly–disassembly were examined in detail. Fibril assembly and disassembly of the collagen induced by slow scans of temperature showed hysteresis. The assembly curve was very sharp whereas the disassembly curve was gradual. Equilibrium centrifugation showed the collagen disassembled from the fibrils to be predominantly monomers. However, unlike the unassembled collagen, the collagen disassembled from fibrils by cooling showed no lag phase in subsequent cycles of fibril assembly. The thermodynamic parameters of fibril growth were derived from a fibril disassembly curve. Fibril growth was weaker for the $NaBH_4$-reduced monomeric collagen than the native crude collagen, perhaps due to the removal of oligomers and the changes in the molecular structure brought by the reduction. The results corroborated the strongly cooperative mechanism for the fibril assembly proposed in the preceding paper (Na et al., 1989).

Preparations of type I collagen from different animal connective tissues invariably contain some oligomers due to the presence of covalent intermolecular cross-links. In subsequent studies of the protein, depending on the particular type of information desired, either the monomeric or the oligomeric species should be selected. For instance, since the collagen in the extracellular matrix is believed to be covalently cross-linked either during or after the process of fibrillogenesis, the fibril assembly of the monomeric collagen appears to be physiologically more interesting. Futhermore, in studying the in vitro fibril assembly of collagen, even a trace amount of the oligomer can affect strongly the kinetics of the reaction and must be removed from the sample for proper data analyses (Na et al., 1986). On the other hand, in determining the locations and identities of the covalent inter-collagen cross-links present in different connective tissues, samples enriched with the oligomers would be desirable. The highly asymmetric shape of the collagen molecule precludes the use of gel and membrane filtration to separate the monomer from the oligomer. A method has been reported which uses differential salt precipitation to separate the collagen into fractions containing different amounts of oligomers (Chandrakasan et al., 1976). This method, in our hands, did not effectively remove the oligomer from the collagen preparation (Na et al., 1986). Many researchers interested in the collagen monomer have resorted to treating the animal with β-aminopropionitrile, a known lathyrogen which inhibits the enzyme lysyl oxidase and prevents the formation of lysine-derived cross-links (Öbrink,

1972; Helseth & Veis, 1981). This method is not always effective, perhaps due to the presence of residual enzyme activity or to the occurrence of other types of covalent cross-links not suppressed by the lathyrogen. Besides, under certain circumstances, the administration of the lathyrogen to animals is impractical.

We recently developed a method of separating the calf skin collagen into two fractions, one enriched with oligomers and the other devoid of such species (Na et al., 1986b). The method takes advantage of the stronger propensity of the oligomer to form fibrils. In this paper, the monomeric and oligomeric collagen preparations were characterized by the determination of several physicochemical properties with the goal of understanding the effects of the inter-collagen cross-links on the structural stabilities of the collagen molecules and the fibrils. The kinetics and equilibrium of the reversible fibril assembly and disassembly of the $NaBH_4$-reduced monomeric collagen were also examined in detail to further understand the mechanism of the reaction.

MATERIALS AND METHODS

$NaBH_4$ was obtained from Sigma Co.[1] The glycerol was Spectranalyzed grade from Fisher.

Preparation of Collagen. The isolation of type I collagen from calf skin and the determination of its concentration are both described in the preceding paper (Na et al., 1989). The collagen obtained from the purification procedure is referred

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[1] Reference of company or produce name does not constitute the endorsement by the U.S. Department of Agriculture over others of similar nature.

to as "crude collagen". Separation of the crude collagen into monomeric and oligomeric fractions was achieved by carrying out partial fibril assembly of the crude collagen. Two different conditions were used. Under the first condition, the crude collagen was dissolved in PS buffer[2] (0.03 M NaP_i/0.1 M NaCl, pH 7) containing 0.9 M glycerol. The concentration of the collagen was adjusted to 2.5 mg/mL, and the solution was incubated at 30 °C. Under the second condition, PS buffer without glycerol was empolyed. The concentration of the collagen was 1.1 mg/mL, and the solution was incubated at 15 °C. Under both conditions, the incubation was allowed to proceed for 48–72 h, and the solution was then centrifuged at 12000g for 30 min to precipitate the fibrils. The fibrils were redissolved in 0.1 M HOAc and dialyzed overnight against 1 mM HOAc. This collagen is referred to as "oligomeric collagen". The collagen remaining in the supernatant was precipitated by adding solid NaCl to 3 m. It was then collected by centrifugation at 12000g for 30 min, redissolved in 0.1 M HOAc, and dialyzed against 1 mM HOAc. This collagen is referred to as "monomeric collagen". Analyses of the near-UV absorption spectra showed that all collagen preparations contained 12 ± 1 tyrosine residues per collagen molecule, indicating that their nonhelical telopeptides were intact (Na, 1988). All collagen preparations were stored under liquid nitrogen until use.

The reduction of collagen with $NaBH_4$ was carried out according to a published procedure (Gelman et al., 1979). The methods of SDS–polyacrylamide gel electrophoresis and optical scanning of the gels have been described in a previous publication (Na et al., 1986).

Measurement of Viscosity. Collagen solution viscosity was measured with Cannon semimicro flow viscometers. Temperature was regulated at 20 °C with a Cannon Model M-1 constant-temperature water bath. The collagen was dissolved in 0.1 N HCl at several different concentrations, and the reduced viscosities were determined.

Unfolding of the Triple Helix. Thermal denaturation of the solubilized collagen was monitored by measuring the hyperchromicity in the far-UV region of the protein in AS buffer (0.01 M NaOAc/0.02 M NaCl, pH 4.0) (Na, 1988). A Perkin-Elmer Lambda-7 UV–visible spectrophotometer was used. Temperature of the collagen solutions was regulated by use of jacketed cells and a jacketed cell holder, both connected to a Neslab Model RTE-8 heating/cooling water bath. A Neslab Model ETP-3 electronic temperature programmer was used to control the water bath to obtain a linear heating rate of 4 °C/h.

Melting of Collagen Fibrils. Thermal melting of the fibrils assembled from the solubilized collagen was probed with a Microcal Model MC-2 differential scanning microcalorimeter. The experimental conditions used were the same as described in the preceding paper (Na et al., 1989). The samples were heated from 4 to 90 °C at three different heating rates. At 25–35 °C, the solubilized collagen self-assembled into fibrils as described in the preceding paper (Na et al., 1989). Continuous heating resulted in the melting of the fibrils at 50–60 °C.

Fibril Assembly and Disassembly. Self-assembly of solubilized collagen into fibrils was carried out in PS buffer. The reaction was monitored by measuring the turbidity of the solution at 350 nm with a Perkin-Elmer Lambda-7 UV–visible spectrophotometer. The spectrophotometer was interfaced to a microcomputer through a RS-232 serial port, and the

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[2] Abbreviations: AS, 0.01 M NaOAc/0.02 M NaCl, pH 4.0; PS, 0.03 M NaP_i/0.1 M NaCl, pH 7.0; Gu-HCl, guanidine hydrochloride.
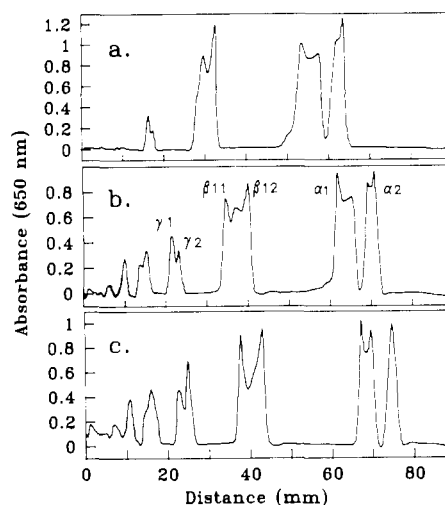

FIGURE 1: SDS–polyacrylamide gel electrophoresis of monomeric (a), crude (b), and oligomeric (c) collagens. Disc gels with 4% polyacrylamide were used. 80–90 μg of collagen was loaded onto each gel. The gels were stained with Coomassie Brilliant Blue R-250 and then scanned with a Shimadzu Model CS-930 scanner at 650 nm. These gels were run separately and not standardized in their run times.

spectral data were recorded in a digital form on diskettes for subsequent analyses. The samples were prepared as described in the preceding paper (Na et al., 1989) and added to the jacketed cuvettes. The kinetics of the fibril assembly–disassembly were examined by a temperature jump method. The jacketed cuvettes in the spectrophotometer were connected to two water baths through two T-shaped stopcocks. One water bath was set at 10 °C and the other at 30°C. Switching the two stopcocks simultaneously allowed the sample temperature to be changed within 30 s between these two settings.

The equilibrium of the fibril assembly–disassembly was studied by scanning the temperature from 4 to 40 °C and back (one cycle) at the slow rates of 1, 2, and 4 °C/h using a single water bath and a temperature programmer in a manner similar to the study of the thermal denaturation of collagen.

Equilibrium Centrifugation. The method of equilibrium centrifugation is described in detail in the preceding paper (Na et al., 1989).

RESULTS

SDS–Polyacrylamide Gel Electrophoresis. The crude, monomeric, and oligomeric collagen preparations were examined with SDS–polyacrylamide gel electrophoresis to determine the nature and degree of covalent cross-links. Figure 1a–c shows optical scans of the gels stained with Coomassie Blue. The crude collagen (Figure 1b) displayed two α bands, two β bands (two α chains cross-linked together), and two γ bands (three α chains cross-linked together). In addition, four bands were noted which migrated slower than the γ bands indicating the presence of intermolecular cross-links. The monomeric collagen (Figure 1a) showed the same α, β, and γ bands as those of the crude collagen. However, the area under the $γ_2$ band decreased substantially, and only a trace amount of the collagen appeared in the region of the high molecular weight bands (>285 000), reflecting the removal of most of the oligomers. The oligomeric collagen (Figure 1c) differed from the crude and monomeric collagens in that a greater percentage of the protein was found under the $γ_2$ and high molecular weight bands.

Solution Viscosity. Solution viscosities of the three collagen preparations were measured in 0.1 N HCl to further characterize the differences in their molecular structures. The results shown in Figure 2 indicate that both the reduced and
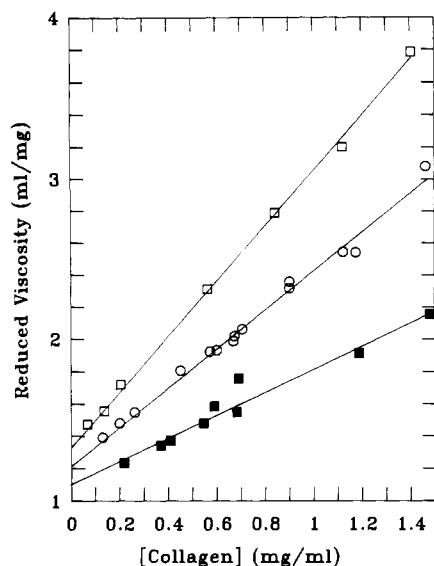
FIGURE 2: Viscosities of different collagen preparations in 0.1 N HCl at 20 °C. The monomeric (■), crude (O), and oligomeric (□) collagen preparations were dissolved in 0.1 N HCl. Extrapolation to zero concentration gave the intrinsic viscosities of 1.08 ± 0.03, 1.21 ± 0.05, and 1.33 ± 0.09 mL/mg for the monomeric, crude, and oligomeric collagen, respectively.
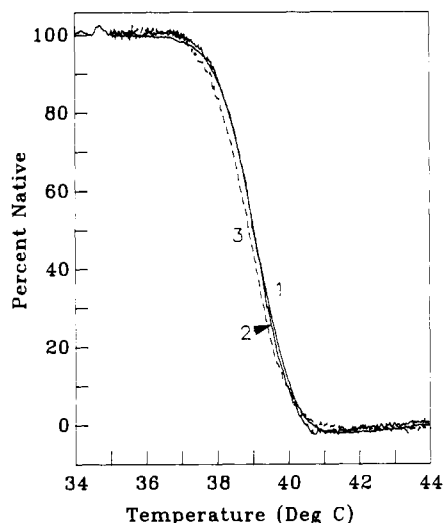

FIGURE 3: Thermal denaturation of calf skin collagen. The reaction was monitored by measuring the hyperchromicity of the collagen at 223 nm. The collagen (0.5 mg/mL) was dissolved in AS buffer. The reference cell contained the same concentration of the collagen but in 6 M guanidine hydrochloride. Curves 1, 2, and 3 represent the crude, oligomeric, and monomeric collagen, respectively.

intrinsic viscosities increased significantly with the amount of oligomers in the collagen preparation. The monomeric, crude, and oligomeric collagens showed intrinsic viscosities of 1.08 $\pm$ 0.03, 1.21 $\pm$ 0.05, and 1.33 $\pm$ 0.09 mL/mg, respectively. The slope of the data in Figure 2 also increased with the amount of oligomers in the solution, from 0.72 for the monomeric collagen to 1.22 and 1.73 for the crude and oligomeric collagens.

Thermal Denaturation of Solubilized Collagen. Thermal denaturation profiles of the three collagen preparations were measured spectrophotometrically in AS buffer (0.01 M Na-OAc/0.02 M NaCl, pH 4.0) as shown in Figure 3. All collagen samples showed sharp and unimodal transition curves. The crude and oligomeric collagen samples had the same denaturation temperature of 39.0 $\pm$ 0.1 °C. The monomeric collagen also showed essentially the same denaturation temperature of 38.8 $\pm$ 0.1 °C.
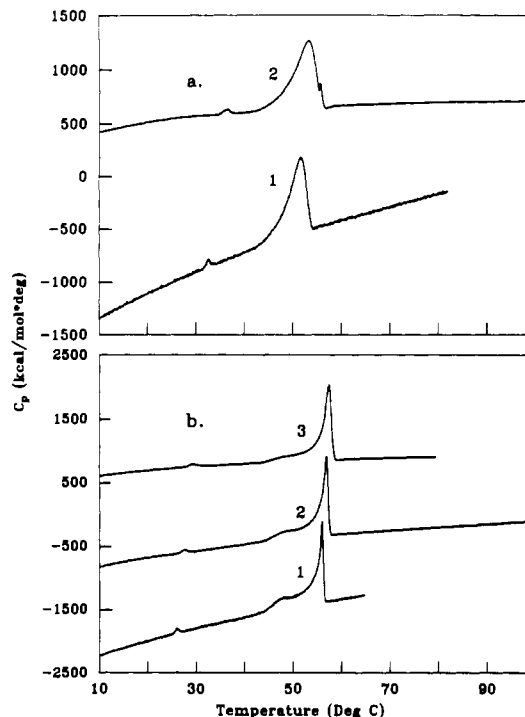

FIGURE 4: Thermal melting of collagen fibrils monitored with differential scanning microcalorimetry. The collagen samples were dialyzed against PS buffer at 4 °C overnight before use. The concentration was 2.478 mg/mL for the monomeric collagen and 2.348 mg/mL for the crude collagen. (a) Thermograms 1 and 2 were obtained from the monomeric collagen at the respective heating rates of 10 and 60 °C/h. (b) Thermograms 1, 2, and 3 were obtained from the crude collagen at the respective heating rates of 10, 30, and 60 °C/h. The small peak located at 25–35 °C of each thermogram corresponds to the heat absorption of the fibril assembly as described in the preceding paper (Na et al., 1989).

Table I: Differential Scanning Calorimetric Study of Fibril Melting[a]

collagen type	heating rate (°C/h)	T_m (°C)	ΔH° [b] ($\times 10^3$) kcal/mol
crude	60	58.2	3.80
	30	57.4	3.97
	10	56.4	3.70
monomeric	60	53.4	3.60
	10	51.6	3.33

[a] Each of the numbers shown here was obtained from a single thermogram. [b] A molecular weight of 285 000 was used in calculating the standard enthalpy changes.

Thermal Melting of Fibrillar Collagen. Thermal melting profiles of the fibrils assembled from the crude and monomeric collagen preparations were measured with a differential scanning microcalorimeter. Figure 4 shows thermograms of the monomeric and oligomeric collagen. A small peak found in each thermogram between 25 and 35 °C corresponds to the assembly of the solubilized collagen into fibrils as reported in the preceding paper (Na et al., 1989). Continuous heating of the sample resulted in the melting of the fibrils as indicated by the much greater heat absorption peak between 40 and 60 °C. For monomeric collagen, a single skewed heat absorption peak was observed with its apex located at 52–53 °C (Figure 4a). For crude collagen, a bimodal melting profile was found (Figure 4b). The first peak was located around 50 °C, similar to that of the monomeric collagen. The second peak was found at a higher temperature of 56–58 °C. The standard enthalpy changes ranged from (3.3–3.6) $\times 10^3$ kcal/mol for the fibrils of the monomeric collagen to (3.7–4.0) $\times 10^3$ kcal/mol for the fibrils of the crude collagen. Both the melting temperatures and standard enthalpy changes are listed in Table I.
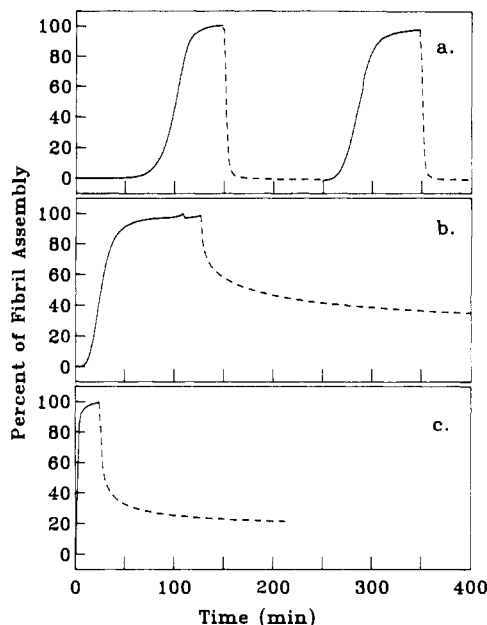
FIGURE 5: Kinetic studies of fibril assembly–disassembly of $NaBH_4$-reduced collagens in PS buffer induced by fast temperature jumps. Panels a, b, and c show, respectively, the fibril assembly-disassembly of the monomeric (1.041 mg/mL), crude (0.168 mg/mL), and oligomeric (0.265 mg/mL) collagen preparations. The fibril assembly reaction was initiated by raising the temperature from 10 to 30 °C (solid curve) whereas the disassembly was induced by lowering the temperature back to 10 °C (dashed curve). The second cycle of fibril assembly and disassembly of the reduced monomeric collagen is also shown in panel a.

Kinetics of Fibril Assembly and Disassembly. The kinetics of fibril assembly and disassembly of the three collagen preparations were examined by a temperature jump method. The fibrils assembled from the three native collagen preparations were less than 20% dissociable by cooling to 10 °C (data not shown). As shown in Figure 5, the reaction became much more reversible if the collagen was reduced with $NaBH_4$ prior to the assembly reaction to prevent the spontaneous formation of covalent inter-collagen cross-links. The degree of reversibility of the fibril assembly reaction of the reduced collagen preparations decreased as the amount of oligomers present increased. By lowering the temperature to 10 °C, the fibrils assembled from the reduced monomeric collagen dissociated completely (>99%) within 10 min (Figure 5a). This was true even in the second cycle and third cycle (not shown) of the fibril assembly reaction. Fibrils assembled from the reduced crude collagen (Figure 5b) and reduced oligomeric collagen (Figure 5c) were only 70–80% dissociable. The disassembly reaction of the latter two collagen preparations consisted of two phases. The first phase was fast and was complete within 30 min. The second phase was very slow and went on for days.

Only the first cycle of the fibril assembly of the reduced monomeric collagen showed a lag phase (Figure 5a). In the subsequent cycles, the solution turbidity rose immediately after the temperature was raised to 30 °C. The growth phase appeared to remain unchanged. The lag phase can be restored, however, by dialyzing the disassembled collagen at 4 °C first against 0.5 M HOAc and then against PS buffer (results not shown). Dialyzing the disassembled collagen against a high ionic strength buffer (0.03 M NaP_i/1 M NaCl, pH 7.0) did not restore the lag phase. Centrifugation (36000g for 30 min), filtration with a 0.22-μm membrane filter (Millipore Millex-GV), and long cooling (72 h at 4 °C) were all found to be ineffective in restoring the lag phase.
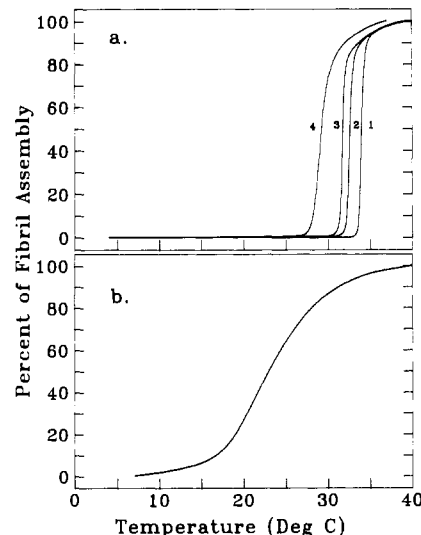

FIGURE 6: Equilibrium studies of fibril assembly and disassembly of $NaBH_4$-reduced monomeric collagen in PS buffer induced by slow scans of temperature. The collagen concentration was 0.25 mg/mL. (a) Curves 1, 2, and 3 depict first-cycle fibril assembly at the heating rates of 4, 2, and 1 °C/h respectively. Curve 4 represents a second-cycle fibril assembly at a heating rate of 6 °C/h. (b) Fibril disassembly at a cooling rate of 2 °C/h. The curve was obtained from the same sample as curve 2 of panel a; the cooling began right after the temperature had reached 40 °C.

Equilibrium of Fibril Assembly and Disassembly. The chemical equilibrium of the reversible fibril assembly–disassembly of the reduced monomeric collagen was examined by slowly scan the temperature between 4 and 40 °C. As depicted in Figure 6, both the assembly and disassembly of fibrils were sigmoidal in shape. The fibril assembly curves for the first cycle of the reaction were very sharp (curves 1–3, Figure 6a). The reaction took place at 30–35 °C with a temperature span of approximately 2 °C. The assembly curve shifted to slightly lower temperatures at slower heating rates. In the second cycle, the fibril assembly curve (curve 4, Figure 6a) shifted to below 30 °C and had a broader temperature span than those of the first cycle.

A fibril disassembly curve of the $NaBH_4$–reduced monomeric collagen is depicted in Figure 6b. The disassembly reaction was gradual and spanned the entire temperature range from 40 to 4 °C. The fibril disassembly curves were found to be independent of the cooling rate, and the disassembly curve of the second cycle was essentially the same as that of the first one (data not shown). For reasons to be given under Discussion, the fibril disassembly curve is believed to reflect the true equilibium of the reaction and was chosen for further equilibrium analysis. Assuming that the turbidity is proportional to the amount of fibrils in the solution and that all of the collagen was assembled into fibrils at the final temperature of the heating cycle (40 °C), then C_r, the amount of collagen left unassembled at time t, can be expressed as

$$C_r = C_0(\tau_f - \tau_t)/(\tau_f - \tau_i) \tag{1}$$

where C_0 is the total concentration of the collagen. τ_i, τ_t, and τ_f are the solution turbidity at the beginning, time t, and the end of the reaction, respectively. Since the unassembled collagen was practically all monomer (Figure 8), the fibril growth constant (K) is equal to the reciprocal of C_r. A van't Hoff plot of the fibril growth constant so obtained is shown in Figure 7a, and the enthalpy changes derived from the slope of the van't Hoff plot are shown in Figure 7b. The fibril growth constants of the reduced monomeric collagen were found to be lower than those reported in the preceding paper
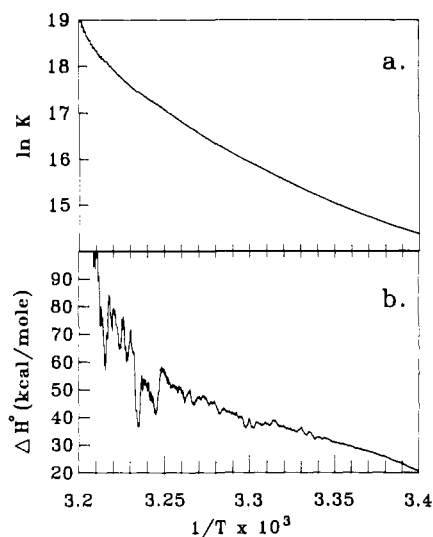
FIGURE 7: van't Hoff plot and the standard enthalpy change of fibril growth for the NaBH₄-reduced monomeric collagen. The calculation was based on the fibril disassembly curve of Figure 6b using eq 1 shown under Results.

for the native collagen derived under the same conditions (Na et al., 1989).

Equilibrium Centrifugation. Equilibrium centrifugation was carried out to examine the state of association of the collagen dissassembled by cooling from the fibrils of NaBH₄-reduced monomeric collagen. As shown in Figure 8, a plot of the logarithm of collagen concentration versus the square of radial distance was quite linear. Linear least-squares fitting of the data gave a molecular weight of 286 800 ± 1600. The inset of Figure 8 shows point molecular weights across the cell calculated from the slope of each of 10 adjacent data points which ranged from 260 000 to 300 000. A few (3-4) data points near the cell bottom ($37.3 \text{ cm}^2 < r^2 < 37.4 \text{ cm}^2$) deviated slightly upward from the straight line. This took place at the centripetal side of the cell bottom ($r^2 = 37.4 \text{ cm}^2$) where the base line started to rise and was observed repeatedly in several runs, suggesting that it is caused by a trace amount of oligomers. However, the small quantity of the aggregate prevented a meaningful analysis of its molecular weight.

DISCUSSION

The method of separating the monomer and oligomer of collagen described here takes advantage of the stronger propensity of the covalently cross-linked oligomer to form fibrils. Such an effect of the oligomer on the kinetics of fibril assembly has been examined in detail in a previous study (Na et al., 1986). Fibril assembly of the monomeric collagen showed a much longer lag time than the crude and oligomeric collagens. More interestingly, the cooperativity of the reaction, measured from the slope of a double-logarithmic plot of the kinetics data according to an equation derived for cooperative nucleation-growth self-associations, increased with the removal of the oligomer from the solution. These results suggested that the cross-linked oligomer either can serve as nucleation centers or can form nucleation centers more easily. Consequently, they become sequestered into the fibril structure during fibril assembly more easily and faster than the monomer. Two fibril assembly conditions were chosen in the separation of the monomer from the oligomer. One was at 30 °C in the presence of glycerol; the latter served as an fibril inhibitor (Na, 1986). The other condition was without glycerol but at a lower temperature of 15 °C. Both conditions are less than optimal for fibril assembly selected for the purpose of giving a weaker fibril
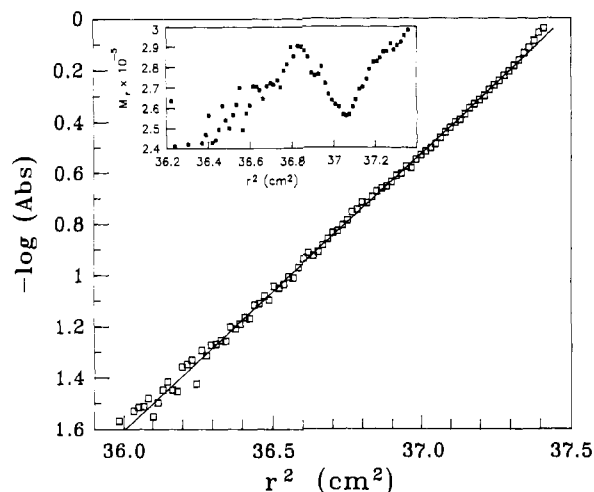

FIGURE 8: Equilibrium centrifugation of collagen disassembled from fibrils at 4 °C. NaBH₄-reduced monomeric collagen was assembled into fibrils by incubating at 30 °C for 2 h. The sample was then cooled to 4 °C and diluted to 0.1-0.2 mg/mL with PS buffer. It was centrifuged at 11 000 rpm and 15 °C for 48 h according to the method described in the preceding paper. In the main figure, the logarithm of the collagen concentration (absorbance at 234 nm) was plotted against the square of radial distance. The straight line represents a linear least-squares fitting of the data which gave an weight average molecular weight of 286 800 ± 1600. The inset shows the point molecular weight derived from the slope of 10 consecutive data points across the cell.

growth (a higher critical concentration) so that some of the collagen will remain unassembled after the incubation. In a previous study, we have shown that the degree of removal of the oligomer is related to the concentration of the crude collagen used in the starting solution; higher concentrations apparently led to fewer oligomers in the supernatant (Na et al., 1986).

The intrinsic viscosity of the collagen solution increased with the amount of the oligomer (Figure 2). This indicated that the oligomer has a more rigid structure than the monomer and/or that the shape of the oligomer is more asymmetrical than that of the monomer. An increased asymmetry of the oligomer could result from stagnation of the cross-linked collagen molecules. Indeed, the most commonly encountered inter-collagen cross-links are between lysine or allysine residues at positions N-9 (N-terminal telopeptide) and 930 and between C-16 (C-terminal telopeptide) and 87 of the α-1 chains on adjacent collagen molecules. They both lead to a 0.4-D overlap and 4-D stagnation (Miller, 1984). The intrinsic viscosity of the collagen solution can, therefore, give a semiquantitative indication of the oligomer content of a collagen sample. The monomeric collagen we obtained had an intrinsic viscosity (1.08 ± 0.03 mL/mg) comparable to that of a monomeric collagen prepared from the skin of the lathyritic rat (1.10 ± 0.03 mL/mg) (Öbrink, 1972).

Intramolecular cross-links were present in all three collagen preparations as evident from the presence of the β and γ bands (Figure 1). This observation suggested that the intramolecular cross-linking was not a factor in the separation and that these collagen preparations differed only in their degrees of inter-molecular and not intramolecular cross-links. One would expect the intramolecular cross-links to stabilize the triple-helical structure of the molecule. The nearly identical thermal denaturation temperatures for the crude, monomeric, and oligomeric collagens shown in Figure 3 suggested again the similar degrees of intramolecular cross-links. Very similar denaturation profiles have previously been observed for the single molecule collagen and high molecular weight collagen

(cross-linked oligomers) extracted from rat tail tendon (Silver & Trelstad, 1980).

Intermolecular cross-links are expected to stabilize the fibril structure. Indeed, animal skins usually show higher shrinkage temperatures[3] after being tanned with chemical agents such as chrome, glutaraldehydes, and polyphenols which cause either covalent or noncovalent cross-links of the collagen. When monitored with a differential scanning microcalorimeter, skins from animals of different age groups also showed different profiles. Those from older animals showed higher melting temperatures, and this has been attributed to an increase with age of the degree of covalent inter-collagen cross-links (Flandin et al., 1984). Interestingly, the thermograms in Figure 4 showed a single skewed peak at 45–55 °C for the fibrils assembled from the monomeric collagen but a bimodal melting transition for those from the crude collagen. An extra peak was observed at a higher temperature of 55–60 °C for the fibrils assembled from the crude collagen. This observation suggested the stabilization of the fibril structure by the covalent inter-collagen cross-links of the oligomers. Multiple melting peaks as 47–50, 53–55, and 57–60 °C have also been reported by Wallace et al. (1986) for different enzyme-treated collagens. They attributed them to the formation of different types of aggregates, including the normally banded fibrils and aberrant structures. The standard enthalpy changes we observed for the melting of the fibrils assembled from the crude collagen [(3.7–4.0) × 10^3 kcal/mol] agreed with the value for the heat-gelled collagen reported by Wallace et al. (1986).

The role of the oligomers in the stability of fibril structure can also be gleaned from the reversibility of fibril assembly of the NaBH$_4$-reduced collagen. Fibrils assembled from the reduced monomeric collagen can be dissociated completely within 10 min after cooling to 4 °C. On the other hand, the disassembly of fibrils assembled from the reduced crude and oligomeric collagens proceeded in two phases. The first phase took place within 30 min after the temperature drop whereas the second one went on for days (Figure 5). These observations suggested that the covelently cross-linked oligomers, perhaps through forming multiple contacts with neighboring collagen molecules, can provide an extra stability to the fibril structure.

The results of the kinetic studies shown in Figure 5a indicated that the disassembled collagen differed from the original unassembled collagen in that it displayed no lag phase in subsequent cycles of fibril assembly. Such an effect has been observed before and was referred to as the "thermal memory effect" (Comper & Veis, 1977a,b; Payne et al., 1986). Evidently, the collagen disassembled from the fibrils by cooling was not completely reversed to its original state. If one attributes the lag phase of the reaction to the formation of nucleation centers, then the disassembled collagen perhaps still contained either some nucleation centers which can be used in the next cycle of fibril assembly or some oligomers which can form nucleation centers more easily. It is interesting that the lag phase can be restored by dialyzing the collagen against 0.5 M HOAc. Dialysis against a high ionic strength neutral buffer (0.03 M NaP$_i$/1 M NaCl, pH 7.0) was ineffective. These results suggested that the residual structure could be stabilized by Schiff base type covalent cross-links, perhaps derived from the residual unreduced allysine residues, which are labile in acid. Also, both low-speed centrifugation (36000g for 30 min) and membrane filtration (0.22 μm) were unable to restore the lag phase, suggesting that the presumed residual structure must be small in size. The amount of the residual structure present in the disassembled collagen solution must also be minuscule; the logarithmic plot of the equilibrium centrifugation data shown in Figure 8 showed only a slight deviation from the best-fitted straight line near the cell bottom, suggesting the presence of no more than a trace amount (<1%) of oligomer in the solution. This small amount of oligomer could have been the cause of the shortened lag phase of fibril assembly. By using the light-scattering technique, Payne et al. (1986) also demonstrated the lack of a significant amount of oligomers in the solution of disassembled fibrils.

Because the kinetic study revealed that the fibril assembly–disassembly of the NaBH$_4$-reduced monomeric collagen was completely reversible, the equilibrium of this reaction was examined in detail. When the temperature was scanned slowly, the fibril assembly and disassembly of the NaBH$_4$-reduced monomeric collagen showed hysteresis; i.e., the assembly and disassembly curves differed from each other. The fibril assembly curve was extremely sharp whereas the disassembly curve was broad and encompassed the entire temperature range from 40 to 4 °C. Also, the assembly curve shifted to slightly lower temperatures at decreasing heating rates whereas the disassembly curve remained essentially unchanged. Such differences suggested that the assembly reaction proceeded through two or more steps and that the initial step was very slow and rate limiting. They also suggested that during the heating the fibril assembly reaction lagged behind the state of equilibrium as dictated by the temperature, perhaps due to the difficulty of forming nucleation centers, whereas the disassembly reaction induced by cooling reflected the true equilibrium of the reaction. The fibril disassembly curve of Figure 6b was, therefore, selected for an equilibrium analysis. The fibril growth constants of the reduced monomeric collagen were found to be lower than those of the native collagen measured under the same conditions (Na et al., 1989). The fibril assembly of the reduced monomeric collagen also showed positive enthalpy changes. However, the enthalpy changes, too, were found to be lower than those of the native collagen. The values are actually closer to those reported by Kadler et al. (1987) for the collagen prepared by treating the procollagen isolated from human fibroblasts with proteinases to remove the propeptides. The cause of these differences is not clear. Reduction with NaBH$_4$ converted the aldehyde group of the allysine residues of the collagen to alcohol. This could have changed the association free energy. The removal of the oligomers could also have contributed to the weakening of the fibril growth.

In summary, the covalent cross-links of collagen found in essentially all connective tissues of adult animals and in most collagen preparations can stabilize the fibril structure. The kinetics and equilibrium properties of the fully reversible fibril assembly and disassembly of the NaBH$_4$-reduced monomeric collagen corroborated the findings with native collagen reported in the preceding paper and reconfirmed the strongly cooperative mechanism of the reaction. The thermodynamic parameters of the fibril growth derived from the disassembly curve conformed qualitatively to those derived from measurements of critical concentrations of the fibril assembly. The weaker fibril growth of the reduced monomeric collagen could reflect the reduction of the allysine residues to hydroxynorlucine residues in the telopeptides and the removal of oligomers.

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[3] Shrinkage temperature is a measurement used by leather chemists to determine the thermal stability of the skin.

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