In Vitro Collagen Fibril Assembly: Thermodynamic Studies

ABSTRACT: The in vitro fibril assembly of calf skin collagen was examined as a function of ionic strength and temperature. In a 0.03 M NaP_i, pH 7.0, buffer, fibril assembly required a minimum critical concentration of collagen. At nearly physiological ionic strengths and temperatures, the critical concentration was less than 1 μg/mL and required a very sensitive method for measurement. Raising the ionic strength of the buffer resulted first in higher and then lower critical concentrations. Raising the temperature led to lower critical concentrations. A van't Hoff plot of the fibril growth constant calculated from the critical concentration gave positive enthalpy changes and positive heat capacity changes which indicate that the fibril growth is driven by both hydrophobic and ionic inter-collagen interactions. Sedimentation equilibrium studies showed the collagen to be monomeric at subcritical concentrations. Differential scanning microcalorimetric studies showed only one very sharp heat absorption peak for the fibril assembly which coincided with the appearance of solution turbidity. Within experimental error, the enthalpy changes of the fibril assembly measured with the microcalorimeter were of the same magnitude as the van't Hoff enthalpy changes. These results are discussed in light of a cooperative nucleation–growth mechanism of collagen fibril assembly proposed earlier [Na, G. C., Butz, L. J., Bailey, D. G., & Carroll, R. J. (1986) *Biochemistry 25*, 958–966].

Type I collagen is synthesized inside fibroblast cells and secreted into extracellular space in the form of procollagen. Subsequent enzymatic cleavages of the N- and C-propeptides yield the collagen monomer which can then self-associate into fibrils to fulfill its biological functions (Miyahara et al., 1982, 1984; Kivirikko & Myllyla, 1984). Collagen fibrils, particularly those in the connective tissues of young animals, can be solubilized under a variety of buffer conditions. The solubilized collagen, when brought back to nearly physiological conditions, can be reconstituted into fibrils of the same morphology, indicating that the collagen molecule itself contains all the necessary structural information for the ordered fibril assembly [for a recent review, see Piez (1984)].

Numerous studies of collagen fibril assembly in vitro have been reported in the past 3 decades aimed at understanding the process of fibrillogenesis in the extracellular matrix. Major interest has been focused on the mechanism of initiation of the reaction, the control of the diameter and length of the fibrils, and the roles of the triple-helical domain and the short nonhelical telopeptides at the carboxy and amino terminals of the molecule. It has been known for decades that the assembly of collagen fibrils, when monitored by measuring the turbidity of the solution, shows two different phases (Bensusan & Hoyt, 1958; Gross & Kirk, 1958; Wood & Keech, 1960; Wood, 1960; Comper & Veis, 1977a,b; Williams et al., 1978; Gelman et al., 1979a). The reaction starts with a lag phase during which the solution does not display any turbidity. Unbanded microfibrils with diameters in the range of 3–15 nm have been observed by electron microscopy during this period (Veis et al., 1979; Gelman et al., 1979a; Na et al., 1986a,b). This is followed by a growth phase in which the turbidity of the solution increases in a sigmoidal manner. Electron microscopy of negatively stained samples taken in this phase showed the appearance of fibrils with periodic 67-nm striations. Such two-phase kinetics led to the suggestion of a cooperative nucleation–growth mechanism for the reaction (Wood & Keech, 1960; Wood, 1960; Cassel et al., 1962). A recent detailed analysis of the kinetic data confirmed that the fibril assembly can be adequately described by a cooperative nucleation–growth association with the initial formation of pentamers as the nucleation centers (Na et al., 1986b).

In a recent equilibrium study, we have shown the requirement of certain minimal concentrations of collagen, referred to as critical concentrations, for the in vitro fibril assembly in buffers containing glycerol (Na et al., 1986a). Furthermore, a velocity sedimentation study showed the collagen to be monomeric at subcritical concentrations. The observation of critical concentrations is consistent with the cooperative self-association mechanism derived from the kinetic data. The results also suggested that the fibril growth is very strong at nearly physiological conditions which rendered the critical concentrations too low to be detected by the method used in earlier studies (Williams et al., 1978; Gelman et al., 1979a,b). By adding glycerol, a fibril assembly inhibitor, to the buffer, the critical concentration of the reaction was brought to an easily measurable range (Na et al., 1986a).

In order to demonstrate the presence of critical concentrations for fibril assembly at nearly physiological conditions and to understand the thermodynamic properties of the reaction, we developed a more sensitive method of measuring the collagen concentration (Na, 1988). By use of this method, the critical concentrations of the fibril assembly reaction were determined as functions of ionic strength and temperature.

The state of association of the collagen at the subcritical concentrations was also probed with equilibrium centrifugation. Furthermore, the timing, rate, and amount of heat exchange of the fibril assembly reaction were examined with differential scanning microcalorimetry. These results are reported in this paper. In the following paper (Na, 1989), the collagen was separated into monomeric and oligomeric fractions and reduced with $NaBH_4$. Several physicochemical properties of these collagen preparations, particularly the reversible fibril assembly–disassembly of the $NaBH_4$-reduced collagen, were examined.

Materials and Methods

UV-grade guanidine hydrochloride (Gu-HCl)[1] was purchased from U.S. Biochemical Corp.[2] All other chemicals used were reagent grade.

Preparation and Concentration Determination of Calf Skin Collagen. Type I collagen was solubilized with 0.5 M HOAc from fresh calf skin obtained from a local slaughterhouse. The protein was salted-out by adding 0.2 volume of 30% NaCl. It was further purified by three cycles of solubilization and salting-out, twice in 0.5 M HOAc and once in 0.05 M NaP_i/0.1 M NaCl, pH 7.0, buffer. Each of the solutions was centrifuged at 15000*g* for 30 min to remove insoluble materials prior to the addition of salt. Salting-out of collagen from the acidic and neutral buffer was done by slow addition of NaCl to 5% and 3 *m*, respectively. The details of the purification procedure have been described in two previous publications (Na et al., 1986a,b). All collagen preparations were characterized by three physicochemical methods. First, the integrity of the triple-helical structure of the molecule was confirmed by the display of a single sharp thermal denaturation transition measured with far-UV differential spectroscopy (Na, 1988,1989). Second, the integrity of the nonhelical telopeptides was ascertained from the number of tyrosine residues per collagen molecule (normally 12 ± 1) determined with near-UV spectroscopy (Na, 1988). Finally, the degree of covalent cross-links of the collagen was probed by SDS–polyacrylamide gel electrophoresis (Na et al., 1986b; Na, 1989).

The concentration of collagen was determined by diluting a small aliquot of the sample with either 6 M Gu-HCl or PS buffer (0.03 M NaP_i/0.1 M NaCl, pH 7.0) and measuring its absorbance at 218 and 203 nm, respectively. The respective absorption coefficients used were 9.43 and 38 $mL \cdot mg^{-1} \cdot cm^{-1}$ (Na, 1988).

Equilibrium of in Vitro Collagen Fibril Assembly. The equilibrium of the in vitro collagen fibril assembly was examined by determining the critical concentration of the reaction. A solution of collagen was prepared by mixing an aliquot of the stock collagen, usually at approximately 10–15 mg/mL and dissolved in 1 mM HOAc, an equal volume of a double-strength fibril assembly buffer, and an appropriate volume of the fibril assembly buffer. The mixture was dispersed by gentle shaking at 4 °C, filtered through a 0.22-μm filter (Millipore), and then centrifuged at 36000*g* for 30 min. The solution was further diluted with the fibril assembly buffer to prepare aliquots of collagen at different concentrations. Fibril assembly was carried out by incubating the aliquots in 1.8-mL microfuge tubes for 24–48 h at the temperature specified. The fibrils were pelleted by centrifugation with a Du Pont-Sorvall SH-MT rotor at 12000*g* for 2 min. The collagen concentration of the solution before the incubation and that of the supernatant were both determined. Their difference was taken as the concentration of fibrils formed in the solution.

The collagen remained unassembled in the supernatant after the removal of the fibrils was tested for its ability to form fibrils. Twelve 2-mL aliquots of collagen at 0.6 mg/mL in PS buffer were prepared and incubated in microfuge tubes at 15 °C for 48 h. The fibrils formed were removed by centrifugation at 12000*g* for 10 min. The concentration of collagen in each supernatant was determined spectrophotometrically as described above. The supernatants were then pooled together. The collagen was salted-out by slow addition of NaCl to 3 *m*, collected by centrifugation, and dispersed in PS buffer. The solution was dialyzed overnight against PS buffer and filtered through a 0.22-μm filter to remove insoluble collagen. After the collagen concentration was adjusted back to 0.6 mg/mL, the solution was divided into 12 0.3-mL aliquots. The fibril assembly of the reconcentrated collagen in these aliquots was examined in the same manner as the initial collagen solution.

Equilibrium Centrifugation of Collagen. Equilibrium centrifugations were performed with a Beckman Model E analytical ultracentrifuge. The cell was assembled from a six-channel centerpiece and sapphire windows. A small sector of the window gaskets was cut off to avoid interference of light passage at the two bottom channels. The cell was loaded with three collagen samples of different concentrations in the range of 0.07–0.2 mg/mL. Centrifugations were carried out at 6000, 9000, and 11 000 rpm. The distribution of collagen within the cell was determined by measuring the absorbance across the cell with an optical scanner, usually at either 232 or 234 nm to give an absorbance of near but less than 1 at the cell bottom. Similar scans were also collected at 250 nm, where the collagen does not absorb appreciably, to be used as the base line. The slowest scan rate, determined to be 0.0542 mm/s through calibrations, was used. The slit in front of the photomultiplier was set at 0.1 mm, and the slit of the monochromator was set at 2 mm. The analog signal of the scanner was sent to a microcomputer where it was converted to digital form by a 12-bit analog-to-digital converter. The analog signal was sampled every 10 ms, and the average of 25 samplings was recorded on a diskette as a data point. Each recorded scan consisted of 1200 data points; 15 scans were collected and further averaged to give the final best-smoothed data. In the particular run shown in Figure 3, three samples of collagen at different concentrations were centrifuged at 10 °C for 3 days. At the end of day 3, the temperature was raised to 12 °C. The centrifugation was allowed to proceed for another 4 days. Scans were collected on each day, and the attainment of equilibrium on day 3 and day 7 was confirmed by the constancy of the scans collected 24 h apart. A partial specific volume of 0.685 mL/g for native collagen was used in calculating the molecular weight (Na, 1986). The densities of the buffer at the temperatures used for centrifugation were determined with an Anton Paar Model DMA-02 precision densitymeter as described in a previous publication (Na, 1986).

Differential Scanning Microcalorimetry. The heat exchange of the fibril assembly reaction was measured with a Microcal Model MC-2 differential scanning microcalorimeter. Collagen samples were adjusted to 2–3 mg/mL and dialyzed overnight at 4 °C against PS buffer before use. The scanning rates used were 10, 30, and 60 °C/h.

[1] Abbreviations: SDS, sodium dodecyl sulfate; Gu-HCl, guanidine hydrochloride; PS, 0.03 M NaP_i/0.1 M NaCl, pH 7.0.

[2] Reference of company or product names does not constitute the endorsement by the U.S. Department of Agriculture over others of similar nature.

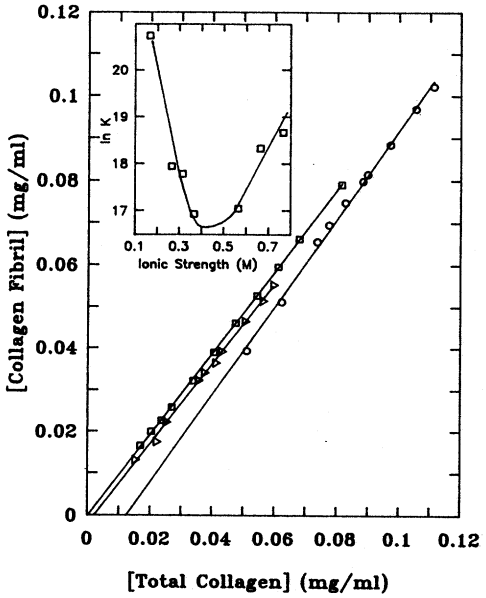

FIGURE 1: Collagen fibril assembly as a function of buffer ionic strength. Concentrations of collagen fibrils were measured in 0.03 M NaP_i, pH 7.0, buffer containing 0.1 (□), 0.3 (○), and 0.7 M (△) NaCl and 0.2, 0.25, 0.5, and 0.6 M NaCl (data not shown). The straight lines are linear least-squares fittings of the data. Their x intercepts gave the critical concentrations shown in Table I. The fibril growth constant (K) was calculated from the reciprocal of the critical concentration based on a molecular weight of 285 000 for the collagen monomer and plotted in the inset as a function of the buffer ionic strength.

Table I: Ionic Strength Dependence of Collagen Fibril Growth[a]

ionic strength (M)	C_r (μg/mL)	$\Delta G°$ (kcal/mol)
0.167	0.28 ± 0.13	−12.5
0.267	4.5 ± 0.3	−10.8
0.317	5.4 ± 0.3	−10.7
0.367	12.4 ± 1.6	−10.2
0.567	10.9 ± 1.3	−10.3
0.667	3.1 ± 0.4	−11.1
0.767	2.2 ± 0.5	−11.3

[a] The buffers contained 0.03 M NaP_i and different concentrations of NaCl and were adjusted to pH 7.0. The temperature was 30 °C.

RESULTS

Ionic Strength Dependence of Fibril Assembly. In order to determine the contribution of ionic interactions toward the self-association of collagen to form fibrils, the critical concentrations of fibril assembly were determined at seven different ionic strengths by increasing the concentration of NaCl in a neutral buffer of 0.03 M NaP_i. Figure 1 shows the results obtained at three different ionic strengths. The concentration of fibrils formed was plotted against the total collagen concentration. The x intercepts of the data gave the critical concentrations listed in Table I. Increasing the concentration of NaCl from 0.1 to 0.3 M resulted in higher critical concentrations for fibril assembly. Further increase of the salt concentration from 0.3 to 0.7 M led to lower critical concentrations. The fibril growth constants (K) at these ionic strengths were calculated from the reciprocal of the critical concentrations. On a logarithmic scale, they showed a V-shaped dependence on the ionic strength as depicted in the inset of Figure 1.

Temperature Dependence of Fibril Assembly. Figure 2 shows the concentration of fibrils formed versus the total collagen concentration measured at four different temperatures of 20, 25, 27.5, and 30 °C. The critical concentrations of the fibril assembly reaction at these temperatures were derived from the x intercepts of the data and are listed in Table II.

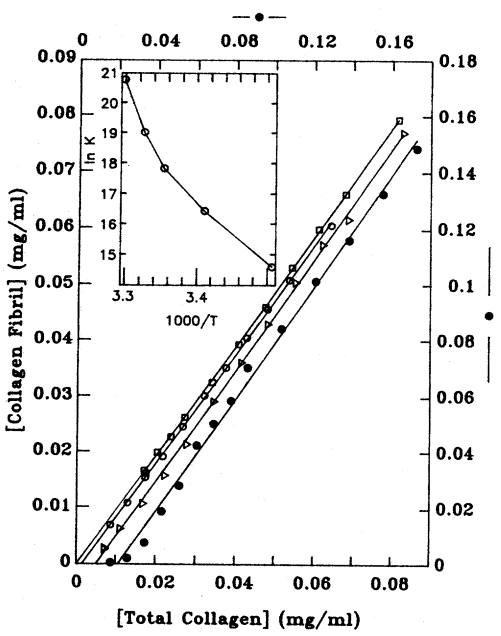

FIGURE 2: Collagen fibril assembly as a function of temperature. Concentrations of collagen fibrils were measured after incubation of the samples in PS buffer at 20 (●), 25 (△), 27.5 (○), and 30 °C (□). The straight lines are linear least-squares fittings of the data. Their x intercepts gave the critical concentrations. The inset at the upper left corner is a van't Hoff plot of the fibril growth constant (the reciprocal of the critical concentration of fibril assembly). The thermodynamic parameters derived from this plot are listed in Table II.

Table II: Temperature Dependence of Collagen Fibril Assembly

temp (°C)	C_r (μg/mL)	$\Delta G°$[a] (kcal/mol)	$\Delta H°$ (kcal/mol)	ΔC_p [kcal/(deg·mol)]
12[b]	130	−8.3	37.9	
20	21.0 ± 1.5	−9.6	48.9	1.7
25	5.1 ± 0.4	−10.6	84.4	9.5
27.5	1.6 ± 0.1	−11.4	123.1	15.5
30	0.28 ± 0.13	−12.5		

[a] The values of $\Delta G°$ were calculated from the critical concentrations of fibril assembly by using a molecular weight of 285 00 for collagen monomer. The values of $\Delta H°$ were obtained from the slopes of the van't Hoff plot for the consecutive data points. [b] The critical concentration at 12 °C was obtained from the collagen concentration at the cell bottom of the equilibrium centrifugation shown in Figure 3.

Critical concentrations in the range of 0.2–21 μg/mL were evident, and they decreased with increasing temperature. The inset at the upper left corner of Figure 2 shows a van't Hoff plot of the polymer growth constants (K). Within the temperature range studied, the reaction showed positive enthalpy changes from 49 to 123 kcal/mol (Table II). The apparent enthalpy changes for the fibril growth increased with increasing temperature, giving positive heat capacity changes in the range of 1.7–15.5 kcal/(mol·K).

Fibril Assembly of Unassembled Collagen in the Supernatant. In the above studies of collagen fibril assembly, due to the presence of critical concentrations, some collagen remained in the supernatant after the removal of the fibrils. The unassembled collagen was subsequently brought to the same concentration as the original collagen solution and examined for its ability to form fibrils. The concentration of the original collagen solution was 0.6 mg/mL. After incubating at 15 °C for 48 h, the samples were centrifuged to precipitate the fibrils. Concentration measurements showed that 0.22 ± 0.02 mg/mL collagen remained in the supernatant. The collagen in the supernatant was then brought back to 0.6 mg/mL through

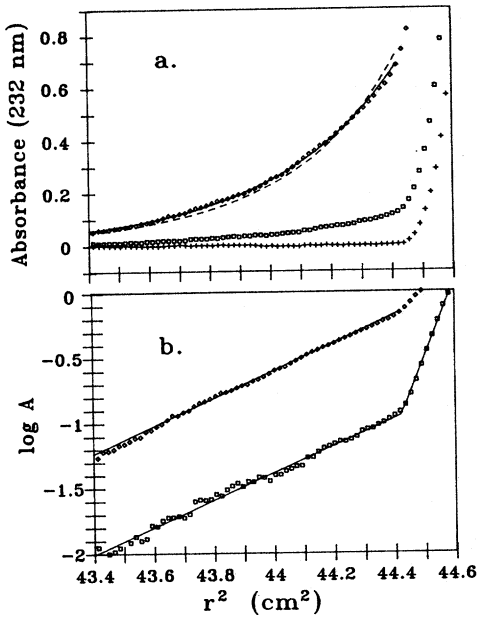

FIGURE 3: Equilibrium ultracentrifugation of native collagen in PS buffer. The rotor speed was 11 000 rpm. The temperature was 10 °C at the beginning of the run and was raised to 12 °C at the end of day 3. (a) Two scans of the sample at 232 nm, one on day 3 at 10 °C (72 ± 3 h) (◊) and another on day 7 at 12 °C (148 ± 3 h) (□). A base-line scan at 250 nm (+) is also shown. Notice the rise in the base-line scan near the cell bottom ($r^2 > 44.42$ cm^2). The solid curve is the nonlinear least-squares fit of the data at 10 °C by a model of 100% collagen monomers (M_r 285 000). The dashed curve is the least-squares fit by a model consisting of 90% monomers and 10% dimers. (b) Plots of the logarithm of the absorbance versus the square of the radial distance. The symbols used were the same as in (a). The straight lines are linear least-squares fitting of the data which gave weight-average molecular weights of 282 200 and 275 900 for the scans on day 3 and day 7, respectively. The data near the cell bottom showed higher slopes. Linear least-squares fitting of the 10 data points collected at 12 °C near the cell bottom gave an apparent weight-average molecular weight of 1 533 000.

salting-out and resolubilization as described under Materials and Methods. The reconcentrated collagen was able to form fibrils under the same conditions, i.e., at 15 °C for 48 h. However, in the second round of fibril assembly, the concentration of collagen remaining in the supernatant was found to be 0.42 ± 0.03 mg/mL, significantly higher than the value of 0.22 ± 0.02 mg/mL determined for the original collagen sample.

Equilibrium Centrifugation of Collagen. Equilibrium centrifugation of collagen was carried out to examine the state of self-association of the protein at and below the critical concentration for fibril assembly. Figure 3a shows the equilibrium distributions of collagen in PS buffer within the centrifuge cell at 10 (◊) and 12 °C (□) measured from the absorbance of the protein at 232 nm. The concentration of the collagen in the cell was higher at 10 °C than at 12 °C. A base-line scan (+) was collected at 250 nm where the collagen does not absorb appreciably. The base-line scan deviated from zero at the cell bottom ($r^2 \geq 44.42$ cm^2). Figure 3b shows plots of the logarithm of the collagen concentration (absorbance) versus the square of radial distance. At $r^2 <$ 44.42 cm^2, where the base line was flat, the plots for both the scans at 10 and 12 °C were linear. Linear least-squares fittings gave apparent molecular weights of 282 200 ± 1400 and 275 900 ± 4000 for 10 and 12 °C, respectively, corresponding closely to the value of 285 000 for collagen monomer derived from the amino acid composition (Miller, 1984). At $r^2 > 44.42$ cm^2 where the base line deviated from zero, the slope of the logarithmic plot also increased sharply. Linear least-squares

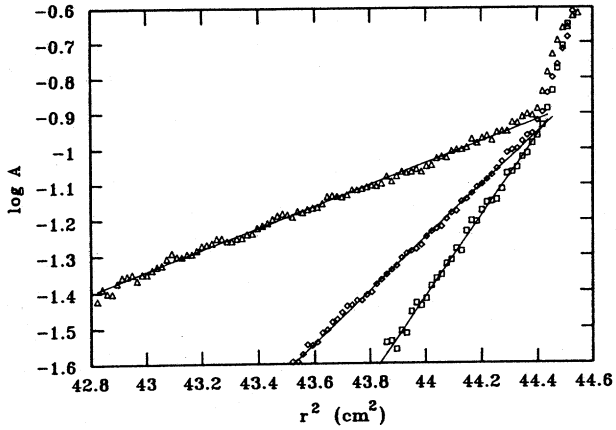

FIGURE 4: Equilibrium centrifugation of collagen in PS buffer at different rotor speeds. The temperature was 12 °C. The absorbance at 232 nm across the cell was recorded with an optical scanner after centrifugation for 72 h. The results of two separate runs at 6000 rpm (△) and 9000 rpm (◊) and the one at 11 000 rpm shown in Figure 3 (□) are shown here. By use of a six-channel centerpiece, each centrifuge run consisted of three samples of collagen at different concentrations. Those shown here were obtained from the middle channel, and each was representative of three samples. The logarithm of collagen concentration (absorbance) was plotted against the square of radial distance. The straight lines are linear least-squares fittings of the data at $r^2 < 44.42$ cm^2. The fittings gave weight-average molecular weights of 276 800 ± 2500 and 288 800 ± 2800 for the runs at 6000 and 9000 rpm, respectively.

fitting of the data points of 12 °C in this region gave an apparent molecular weight of 1 533 000 ± 38 000.

The concentration distribution of collagen at 10 °C was fitted by a model consisting of collagen monomer and dimer at different percentages using a nonlinear least-squares method. The solid curve in Figure 3a depicts the best fit of the data by a model consisting of 100% collagen monomer. The sum of square of deviation (SSD) between the experimental data and the theoretical curve was 0.0019. Introduction of an increasing amount of dimers into the model resulted in deterioration of the fit. This is evident from the dashed curve of Figure 3a, which represents the best fit of the data by a heterogeneous model of 90% monomer and 10% dimer. The SSD value of this fit was 0.0234. The best fit of the data by a heterogeneous model of 95% monomer and 5% dimer (curve not shown) had a SSD value of 0.0088.

Equilibrium Centrifugation of Collagen at Lower Rotor Speeds. Equilibrium centrifugations were carried out at two rotor speeds of 6000 and 9000 rpm. As shown in Figure 4, logarithmic plots of the scans collected at 6000 rpm (△) and 9000 rpm (◊) remained linear at $r^2 < 44.42$ cm^2, suggesting the presence of a single species. Linear least-squares fittings of the data gave apparent molecular weights of 276 800 ± 2500 and 288 700 ± 2800 for the runs at 6000 and 9000 rpm, respectively. They correspond closely to the value obtained at 11 000 rpm (275 900 ± 4000) and the known molecular weight of collagen monomer (285 000) (Miller, 1984). On the other hand, at $r^2 > 44.42$ cm^2, the slopes of the three sets of data collected at 6000, 9000, and 11 000 rpm appeared to be quite similar and not much affected by the change in rotor speed.

Differential Scanning Microcalorimetric Study of Fibril Assembly. The timing, rate, and amount of heat exchange of the fibril assembly reaction were monitored with a differential scanning microcalorimeter. Collagen samples equilibrated in PS buffer were heated in the calorimeter from 4 to 40 °C at three different rates. A single very sharp heat absorption peak was observed between 26 and 30 °C as evident

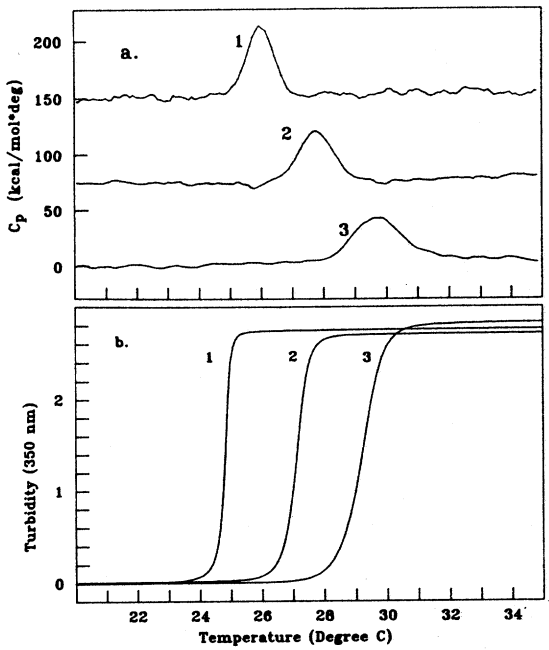

FIGURE 5: Differential scanning microcalorimetric study of collagen fibril assembly. Collagen was dialyzed against PS buffer before use. The final protein concentration was 2.348 mg/mL. The samples were heated from 4 to 40 °C. (a) Thermograms 1, 2, and 3 were obtained at the heating rates of 10, 30, and 60 °C/h, respectively. (b) Concurrent turbidimetric study of the fibril assembly of the same collagen solution. Curves 1, 2, and 3 were obtained at the respective heating rates of 10, 30, and 60 °C/h.

Table III: Differential Scanning Calorimetric Study of Collagen Fibril Assembly[a]

collagen type	heating rate (°C/h)	t^b (°C)	$\Delta H°$ (kcal/mol)
crude	60	29.9	92
	30	27.9	74
	10	26.1	93
monomeric[c]	60	36.6	87
	10	32.6	91

[a] Each of the numbers shown here was obtained from a single thermogram. [b] This is the temperature at the apex of the endothermic peak shown in Figure 5. [c] The method of preparation of the monomeric collagen and several of its physicochemical properties are described in the following paper (Na, 1989).

from the three thermograms shown in Figure 5a. The apex of the peak shifted to slightly lower temperatures as the heating rate was reduced. Concurrent turbidimetric studies of the same sample under the same heating conditions with a UV/visible spectrophotometer indicated that the heat absorption coincided with the emergence of the solution turbidity (Figure 5b). The enthalpy changes of the fibril assembly reaction determined from the areas under the endothermic peaks were in the range of 74–93 kcal/mol with no clear trend of dependence on the transition temperature (Table III). The fibril assembly of a monomeric collagen (Na, 1989) was also examined with differential scanning microcalorimetry (thermograms not shown) and found to be quite similar to that of the crude collagen except the transition temperature was somewhat higher. The transition temperatures and enthalpy changes of fibril assembly of the monomeric collagen at the heating rates of 10 and 60 °C/h are also listed in Table III.

Discussion

The self-assembly of type I collagen into fibrils has been under continuous investigation for over 3 decades. However, the mechanism of the reaction still remains obscure today. Many recently reported studies focused on the lag phase of the reaction, particularly with the use of the dynamic light-scattering and X-ray scattering techniques, to probe the initial steps of the reaction (Silver et al., 1979; Silver & Trelstad, 1980; Silver, 1981; Gelman & Piez, 1980; Bernengo et al., 1983; Suarez et al., 1985; Payne et al., 1986). From these studies, a major disagreement emerged regarding whether a significant amount of collagen oligomers forms as an intermediate during the lag time of fibril assembly. Three of these groups reported self-associations of collagen during the lag phase (Gelman & Piez, 1980; Silver et al., 1979; Silver & Trelstad, 1980; Silver, 1981; Suarez et al., 1985). They suggested that the self-associations lead to the formation of small-size aggregates which would not cause any measurable change in the solution turbidity. Silver and Trelstad concluded that the collagen molecules first self-associate into 4-D staggered dimers and trimers (Silver & Trelstad, 1980; Silver, 1981). On the other hand, Gelman and Piez suggested the formation of aggregates containing 5–100 monomers. Two other laboratories reported results completely different from those described above. Using both the dynamic light-scattering and electric birefringence techniques, Bernengo et al. (1984) could not detect the formation of any significant amount of collagen oligomers during the lag phase of fibril assembly. This was confirmed recently by a similar light-scattering study reported by Payne et al. (1986). The former group attributed the turbidity lag to an intramolecular event, perhaps in the form of a conformational change leading to a stiffening of the molecule. However, direct evidence for such a structural change has not been forthcoming. Furthermore, recent kinetic studies indicated that the length of the lag phase is inversely related to both the concentration and the oligomer content of the collagen (Na et al., 1986b; Na, 1989), suggesting that the lag phase is due largely to intermolecular associations.

In view of the above conflicting results and the inherent pitfalls in the use of the dynamic light scattering to probe transient states of protein self-associations, we decided to take a different approach to study the mechanism of collagen fibril assembly, namely, by examining the chemical equilibrium and probing the energetics of the reaction. The equilibrium properties of the reaction, although they do not specify directly an association pathway, can be used as guidelines in formulating the mechanism of the association. A number of reports have appeared in the past suggesting a cooperative nucleation–growth mechanism for the reaction (Wood, 1960; Cassel et al., 1962; Cooper, 1970; Wallace & Thompson, 1983). Most of these reports were based on kinetic evidence. The reaction has not been examined critically with an equilibrium approach. The equilibrium study we reported recently (Na et al., 1986a) and that in this paper demonstrate that the fibril assembly reaction displays the classic critical concentration phenomenon observed typically in strongly cooperative self-association systems such as the assembly of actin filaments (Oosawa & Kasai, 1962, 1971) and microtubules (Gaskin et al., 1974; Lee & Timasheff, 1977). In the previous report, glycerol was added to the buffer in order to raise the critical concentration to an easily measurable level (Na et al., 1986a). In the present paper, we demonstrated that, by employing a more sensitive method of concentration measurement, critical concentrations of fibril assembly at the microgram per milliliter and submicrogram per milliliter levels can be observed under nearly physiological conditions. Recently, Kadler et al. (1987) also reported the observation of critical concentrations for the in vitro fibril assembly of type I collagen. Their collagen was obtained from the procollagen of human fibroblasts. The propeptides were removed in vitro with propeptide proteinases,

and radioactive labeling allowed the measurement of low concentrations of collagen.

While a constant concentration of collagen was found unassembled after incubation, the possibility remained that this collagen actually represented a subgroup of the total collagen which was unable to form fibrils. To rule out this possibility, the collagen that remained unassembled in the supernatant was brought back to the same concentration as the original solution and tested for the ability to form fibrils. The results showed clearly that the unassembled collagen in the supernatant, once concentrated to above the critical concentration of fibril assembly, can self-associate into fibrils. It is worth noting, however, that the critical concentration of the second fibril assembly reaction is significantly higher than that of the first one measured under the same conditions. This difference is most likely due to the presence of a smaller amount of oligomers in the reconcentrated collagen than the original one. As studied in detail in the following paper (Na, 1989), collagen oligomers have a stronger propensity to form fibrils. Consequently, the assembled fibrils became enriched with the oligomer whereas the unassembled collagen in the supernatant was devoid of the oligomer. Since the inverse of the critical concentration can be taken as the fibril growth constant, the removal of oligomers from the solution could have weakened the fibril growth and led to the higher critical concentration observed. Indeed, fibril growth constants of the monomeric collagen reported in the following paper (Na, 1989) are weaker than those for the native collagen shown in this paper.

To probe the driving forces of collagen fibril assembly, we measured the critical concentration of fibril assembly as a function of ionic strength and temperature. The results in Figure 1 showed that the fibril growth constant first decreased with increasing ionic strength of the buffer. This weakening of fibril growth is probably due to the Debye–Hückel screening effect and suggests the formation of ion pairs in the association.

At ionic strengths above 0.367 M, the critical concentration started to decrease, and the fibril growth became stronger again. This resulted in an overall V-shaped dependence of the fibril growth constant on the ionic strength (inset of Figure 1). Microscopic examinations confirmed that the aggregates formed under higher concentrations of NaCl were indeed fibrillar rather than amorphous. Interestingly, a similar V-shaped dependence of the rate of fibril assembly (reciprocal of the reaction half-time) of pN-collagen, a collagen with the N-terminal propeptides remaining on the molecule, has been reported by Miyahara et al. (1983). The minimum of the kinetic curve was also found near 0.35 M ionic strength. The enhancement of fibril growth by high concentrations of NaCl is most likely due to a nonspecific preferential exclusion of the salt from the surface area of the collagen molecule. Preferential exclusions of NaCl by bovine serum albumin and lysozyme at high salt concentrations have been reported by Arakawa and Timasheff (1982). The mutual exclusion can lead to higher chemical potentials of both the protein and the salt and can provide a driving force for a number of phase separation phenomena of proteins including salting-out, crystallization, and enhancement of subunit self-association (Na & Timasheff, 1981; Arakawa & Timasheff, 1982; Na, 1986). During purification, collagen is usually salted-out from neutral buffers at 4 °C by 1–3 M NaCl, indicating that the salt at high concentrations is preferentially excluded by the collagen. The mutual exclusion probably remained effective at the higher temperatures used in the fibril assembly but manifested itself in an enhancement of the ordered self-association of the collagen into fibrils.

The temperature dependence study of fibril assembly showed that the reaction is favored by higher temperatures. The van't Hoff plot in Figure 2 gave positive enthalpy changes in the range of 49–123 kcal/mol and positive heat capacity changes. The self-association reaction is, therefore, entropy-driven, which has been found true in the assembly of many other biological supramolecular structures such as actin filaments (Kasai, 1969), tobacco mosaic virus protein (Banerjee & Lauffer, 1966), and microtubules (Lee & Timasheff, 1977). The positive enthalpy changes indicated the involvement of inter-collagen hydrophobic interactions in the fibril growth. Hydrophobic interactions are known to be accompanied by positive enthalpy changes and negative heat capacity changes whereas ionic interactions are usually characterized by positive enthalpy changes and positive heat capacity changes (Timasheff, 1973; Tanford, 1973; Cantor & Schimmel, 1980). It appears that the ionic interaction and the hydrophobic effect balanced one another and resulted in positive enthalpy changes but positive heat capacity changes for the reaction.

The enthalpy changes we observed for the fibril assembly were higher than those reported by Kadler et al. (1987). The cause of the difference is not clear. One possible factor is that their collagen was extracted from fibroblasts and none of its lysine groups should have been converted to allysine. Since the collagen used in this study was purified from calf skin, several lysine and hydroxylysine residues have been converted into allysine and hydroxyallysine residues by lysyl oxidase in the extracellular matrix and are thus capable of forming covalent cross-links. Interestingly, the enthalpy changes of fibril assembly of the $NaBH_4$-reduced collagen shown in the following paper (Na, 1989), where the allysine groups had been reduced to hydroxynorleucine, were closer to the values reported by Kadler et al. (1987).

The effects of temperature, salt types, and ionic strength on the kinetics of collagen fibril assembly (reaction half-time) have been examined by Williams et al. (1978). The effects of these solution variables on the chemical equilibrium of fibril growth reported in the current paper are parallel to their effects on the kinetics of the reaction reported by Williams et al. (1978).

The equilibrium distributions of collagen in the centrifugal field shown in Figures 3 and 4 revealed again the existence of critical concentrations of fibril assembly and their dependence on temperature. In Figure 3a, the concentration of collagen at 12 °C was found to be much lower than that at 10 °C across the cell. The scan collected at 10 °C showed the maximum concentration of collagen to be 0.75 mg/mL at the cell bottom. This should be equal to the critical concentration of fibril assembly at the temperature. After the temperature was raised to 12 °C, the collagen concentration exceeded the critical concentration of fibril assembly, and the collagen started to form fibrils. This resulted in depletion and redistribution of the collagen in the centrifuge cell until the maximum concentration of collagen at the cell bottom decreased to 0.13 mg/mL which should correspond to the critical concentration of fibril assembly at 12 °C. The formation of fibrils was indicated by a small amount of white precipitate observed at the cell bottom at the end of the run. In Figure 4, nearly identical concentrations of collagen were found at the cell bottom of three separate runs at the same temperature despite the fact that they were started at different loading collagen concentrations and were spun at different rotor speeds.

The unassembled collagen at subcritical concentrations must remain mostly monomeric to justify the use of its concentration

to calculate the apparent polymer growth constant (Oosawa & Asakura, 1975; Timasheff, 1981). The results of a velocity sedimentation study we reported earlier (Na et al., 1986a) demonstrated that the collagen in the glycerol-containing buffer was monomeric at subcritical concentrations of fibril assembly. The same point was reconfirmed, this time in PS buffer, by the equilibrium centrifugations shown in Figures 3 and 4. The linearity of the data points in Figure 3b suggested the presence of only a single species. Linear least-squares fittings gave weight-average molecular weights of 282 200 ± 1400 and 275 900 ± 4000 for the data at 10 and 12 °C, respectively. The data collected at 10 °C were further fitted by a model with different amounts of monomer and dimer. Inclusion of collagen dimers caused a deterioration of the data fit as evident from the dashed curve of Figure 3a.

As stated under Materials and Methods, the slit width of the scanner photomultiplier was set at 0.1 mm. The base-line scan shown in Figure 3a began to rise sharply as soon as the front edge of the slit moved from the solution to the cell bottom (r = 6.67 cm). The same rise in absorbance near the cell bottom was also observed when the cell was filled with only water. Consequently, the data collected within approximately one slit width from the cell bottom were useless. In Figure 3b, the logarithmic plots of the scans at both 10 and 12 °C rose sharply near the cell bottom ($r^2 > 44.42$ cm^2). Linear least-squares fitting of the data points collected at 12 °C in this region gave an apparent molecular weight of 1 533 000 ± 38 000. Since these data points are located at the region where the base line deviated from zero, the high slope is most likely an artifact due to the finite slit width rather than an indication of the presence of high molecular weight aggregates. The finite slit width in effect created a small "blind spot" near the cell bottom where a small amount of high molecular weight species (MW >1 500 000) could distribute themselves and escape detection by the scanner. To alleviate this problem, equilibrium centrifugations were carried out at two lower speeds of 6000 and 9000 rpm. At lower rotor speeds, one would expect the high molecular weight species, if any, to distribute themselves more centripetally and away from the "blind spot" at the cell bottom. The linearity of the data at the lowest rotor speed of 6000 rpm shown in Figure 4 and the agreement of the molecular weight derived from this plot with that of collagen monomer provided an even stronger argument against the presence of a significant amount of oligomers in the solution.

The lack of a measurable amount of oligomers in the sample was at first surprising, knowing that the collagen preparation did contain some covalently cross-linked oligomers (Na et al., 1986b, Na, 1989). Indeed, when studied in an acidic buffer, the collagen sample showed a measurable amount of oligomers (G. C. Na, unpublished results). Upon further considerations, it became clear that in the fibril assembly buffer most of the covalently cross-linked collagen oligomers, because of their stronger propensities to form fibrils, should have formed fibrils and been pelleted to the cell bottom (Na, 1989). Therefore, the collagen shown in Figures 3 and 4 should have most of the covalently cross-linked oligomers removed (Na, 1989). A self-association of collagen into aggregates with the size of tetramer to pentamer has been reported by Yuan and Veis (1973) based on an equilibrium centrifugation study under conditions leading to incipient fibril assembly. This piece of data has been referenced from time to time as evidence for the formation of collagen oligomers as an initial step of fibril assembly. In their study, the presence of the aggregates was indicated by a sharp rise of the solution absorbance within 0.15 mm from the cell bottom similar to the one shown in Figure 3. Since base lines were not recorded in their study, the collagen oligomers they reported could also have been contributed by the above-mentioned artifact.

The results of the differential scanning microcalorimetric study of fibril assembly shown in Figure 5 indicated only one very sharp heat absorption peak which coincided with the appearance of solution turbidity. Since no other heat absorption or release was observed, the result suggested that the fibril assembly reaction is strongly cooperative and that no reactions with significant enthalpy changes, either intramolecular or intermolecular, take place prior to the growth of the fibrils. This is consistent with the equilibrium centrifugation results in Figures 3 and 4 and the results of the light-scattering studies reported by Bernengo et al. (1983) and by Payne et al. (1986); neither group could detect the formation of collagen oligomers in the lag phase, i.e., prior to the appearance of solution turbidity. Within experimental error, the enthalpy changes of fibril assembly obtained from the calorimeter agreed with the enthalpy changes derived from the van't Hoff plot of the critical concentrations (Table III). However, the van't Hoff enthalpy changes were subject to great uncertainties at the temperature range where the calorimetric enthalpy changes were measured (26–37 °C). Above 30 °C, the critical concentration of the fibril assembly was so low (<0.27 μg/mL) that it reached the limit of the sensitivity of the collagen measurement technique used here. The data certainly do not lend themselves to a firm conclusion regarding the parity of the enthalpy changes determined from the two different approaches and, therefore, cannot be used as evidence either for or against the involvement of more than one step and the formation of intermediate species in the fibril assembly.

It should be stressed that the fibril assembly of the native collagen under examination here is irreversible by cooling due to the spontaneous formation of covalent inter-collagen cross-links. It is, therefore, quite intriguing that critical concentrations were observed for this reaction just as in the reversible fibril assembly of the $NaBH_4$-reduced collagen reported in a previous paper (Na et al., 1986a) and in the following paper (Na, 1989). The presence of critical concentrations irrespective of the reversibility of the reaction suggests that the collagen monomer was not in direct equilibrium with the fibrils. Otherwise, it would have been trapped completely in the fibrillar state by the covalent cross-links. One possible explanation is that the collagen monomers first polymerize reversibly into an intermediate species. The intermediate species further self-associates, either concurrently or subsequently, into fibrils. Under such a scheme, one could envisage a condition where all the intermediate species have turned into fibrils leaving some collagen monomers in the solution. These monomers are unable to initiate the formation of new intermediate species because they are below the critical concentration and unable to polymerize onto the existing fibrils because it is a prohibited route. If this model is correct, then the polymer growth constant derived from the critical concentration measurements should correspond to the growth of the intermediate species and not the fibril.

The chemical equilibrium and thermodynamic parameters of the fibril assembly reaction reported here, although they do not specify an association pathway themselves, are useful as a test stone to verify the mechanisms proposed on the basis of other evidence. For instance, the observation of critical concentrations indicated that the reaction is strongly cooperative; i.e., a weak self-association must precede a much stronger one. This type of cooperativity has been observed in

the assembly of a number of biological supramolecular structures including the polymerization of G-actin into filaments (Oosawa & Kasai, 1962) and the assembly of tubulin into microtubules (Gaskin et al., 1974). Cooperativity is recognized as one of the means used by these biological systems to restrict the number of growing sites and thereby generate a small number of very large biomolecular assemblies rather than a large number of small aggregates. For such systems, an acceptable association mechanism must contain certain schemes to support such a cooperativity. Our equilibrium data refuted the mechanism proposed by Silver and co-workers with the initial formation of substantial amounts of collagen dimers and trimers (Silver et al., 1979; Silver & Trelstad, 1980; Silver, 1981). The accumulation of a substantial amount of oligomers, particularly the dimer and trimer, points to a sequential association with equal or comparable free energy changes for each step of the association (isodesmic) (Na & Timasheff, 1985). Systems with such an association scheme, exemplified by the vinblastine-induced tubulin self-association (Na & Timasheff, 1980), should not display any critical concentrations. On the other hand, a strongly cooperative self-association exhibiting critical concentrations should not accumulate any significant amount of oligomers smaller than the nucleation center (Oosawa & Asakura, 1975; Timasheff, 1981; Na, 1986a). Even the nucleation centers are expected to be too few and too temporal to be detected by the conventional physicochemical methods such as light scattering used in previous studies (Bernengo et al., 1983; Payne et al., 1986) and equilibrium centrifugation used in the current study. This should not be surprising considering the extremely long threadlike structure of the collagen fibrils. Indeed, in the cooperative assembly of two other well-known biological supramolecular structures, namely, the formation of actin filaments and microtubules, intensive studies have not disclosed any reliable direct evidence for the nucleation centers of the reactions.

In summary, the in vitro fibril assembly of type I collagen displays the classic critical concentration phenomenon at nearly physiological conditions. The results, being consistent with the data we reported earlier (Na et al., 1986a,b), strongly suggest a cooperative nucleation–growth mechanism for the fibril assembly reaction. Ionic strength and temperature dependence of the fibril growth constant indicated that the fibril assembly reaction is entropy-driven and involves both hydrophobic and ionic interactions.

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