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Assembly of Collagen Fibrils *de Novo* by Cleavage of the Type I pC-Collagen with Procollagen C-Proteinase

ASSAY OF CRITICAL CONCENTRATION DEMONSTRATES THAT COLLAGEN SELF-ASSEMBLY IS A CLASSICAL EXAMPLE OF AN ENTROPY-DRIVEN PROCESS*

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Type I procollagen was purified from the medium of cultured human fibroblasts incubated with ^{14}C-labeled amino acids, the NH_2-terminal propeptides were cleaved with procollagen N-proteinase, and the resulting pC-collagen was isolated by gel filtration chromatography. pC-collagen did not assemble into fibrils or large aggregates even at concentrations of 0.5 mg·ml^{-1} at 34 °C in a physiological buffer. However, cleavage of pC-collagen to collagen with purified C-proteinase (Hojima, Y., (1985) *J. Biol. Chem.* 260, 15996–16003) generated fibrils that were visible by eye and that were large enough to be separated from solution by centrifugation at 13,000 × *g* for 4 min. With high concentrations of enzyme, the pC-collagen was completely cleaved in 1 h, and turbidity was near maximal in 3 h, but collagen continued to be incorporated in fibrils for over 10 h.

Because the pC-collagen was uniformly labeled with ^{14}C-aminoacids, the concentration of soluble collagen and, therefore, the critical concentration of polymerization were determined directly. The critical concentration was independent of the initial pC-collagen concentration and of the rate of cleavage. The critical concentration decreased with temperature between 29 and 41 °C and was 0.12 ± 0.06 (S.E.) μg·ml^{-1} at 41 °C. The thermodynamic parameters of assembly were essentially independent of temperature in the range 29 to 41 °C. The process was endothermic with a ΔH value of +56 kcal·mol^{-1}, but entropy driven with a ΔS value of +220 cal·K^{-1}·mol^{-1}. The Gibbs energy change for polymerization was −13 kcal·mol^{-1} at 37 °C.

The data demonstrate, for the first time, that type I collagen fibril formation *de novo* is a classical example of an entropy-driven self-assembly process similar to the polymerization of actin, flagella, and tobacco mosaic virus protein.

Type I collagen in the form of microscopic fibrils or thicker fibers and fiber bundles is a major constituent of the extracellular matrix of most complex organisms. The fibrils are generally assumed to form by self-assembly of the type I collagen monomer. The assembly of collagen fibrils was extensively studied in the past with collagen extracted from tendon or skin with cold acidic solutions and then reconstituted into fibrils by neutralizing and warming the solutions (Gross and Kirk, 1958; Wood, 1960; Cooper, 1970; Leibovich and Weiss, 1970; Comper and Veis, 1977; Williams *et al.*, 1978; Gelman *et al.*, 1979a, 1979b; Silver *et al.*, 1979; Helseth and Veis, 1981; Silver, 1982; Farber *et al.*, 1986; Holmes *et al.*, 1986; Na *et al.*, 1986a, 1986b). However, the fibrils formed with extracted collagen in physiological buffers tend to be narrow in diameter and lack the tightly structured appearance of fibrils *in situ* (see Cooper, 1970; Miyahara *et al.*, 1982). Also, extracted collagen does not reproducibly form fibrils at temperatures above 35 °C (see Cooper, 1970). For these and related reasons, the thermodynamics of collagen self-assembly have not been examined as thoroughly as several other biological polymerization processes such as the polymerization of tobacco mosaic virus protein (Lauffer, 1975), of actin (Oosawa and Asakura, 1975; Zimmerle and Frieden, 1986), and of flagella (Gerber *et al.*, 1973).

Here we used a novel system for studying the assembly of collagen fibrils *de novo* in which collagen is generated enzymically under physiological conditions from an intermediate in the normal processing of type I procollagen to type I collagen (Miyahara *et al.*, 1982, 1984). The system made it possible to measure the critical concentration of the monomer and thereby demonstrate that polymerization under physiological conditions is an entropy-driven process with thermodynamic parameters similar to those for other protein polymerizations.

MATERIALS AND METHODS

Preparation of Procollagen—^{14}C-Labeled type I procollagen was purified from the medium of cultured fibroblasts from normal human skin. Fibroblasts from two normal individuals were used: GM 3349 (Human Genetic Mutant Cell Repository), a 10-year-old black male, and JIMM-86, a 41-year-old white female. Cells were grown to confluency in 40, 175-cm^2 cell-culture flasks with DMEM[1] containing 10% fetal bovine serum. To label procollagen, cells were first washed with phosphate-buffered saline and then incubated for two consecutive periods of 24 h in DMEM containing 1 μCi·ml^{-1} of a uniformly labeled mixture of L-^{14}C-aminoacids (supplied by ICN Biochemicals in 2% ethanol), 25 μg·ml^{-1} L-ascorbic acid, and no fetal bovine serum. The medium was harvested after each 24-h period. Finally, the cells

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[1] The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; N- and C-, amino- and carboxyl-terminal, respectively; pC-collagen, intermediate in the conversion of the procollagen to collagen containing the COOH-terminal propeptides but not the NH_2-terminal propeptides; pN-collagen, intermediate in the conversion of procollagen to collagen containing the NH_2-terminal propeptides but not the COOH-propeptides; pC-α chains, polypeptides of pC-collagen; SDS sodium dodecyl sulfate.

were incubated for a third 24-h period in DMEM containing unlabeled amino acids and 25 $\mu g \cdot ml^{-1}$ ascorbic acid. Immediately after medium was removed from the cells, it was cooled on ice, 0.1 volume of 250 mM EDTA, 0.2% NaN_3, 1 M Tris-HCl buffer, pH 7.4, at 20 °C was added, and proteins were precipitated with 176 $mg \cdot ml^{-1}$ ammonium sulfate (Fiedler-Nagy *et al.*, 1981; Peltonen *et al.*, 1980). The precipitates from the three samples of medium were combined and resuspended in storage buffer that consisted of 0.4 M NaCl, 0.01% NaN_3, 0.1 M Tris-HCl buffer, pH 7.4, at 20 °C. The type I procollagen was purified on two successive DEAE-cellulose chromatography columns (Fiedler-Nagy *et al.*, 1981; Peltonen *et al.*, 1980). Type I procollagen was eluted from the second DEAE-cellulose column at 0.025 M NaCl. The pooled fractions were dialyzed against storage buffer, concentrated by pressure ultrafiltration using a membrane (Amicon YM-100) with a 10^6-dalton molecular mass cut-off, and stored at −15 °C. Procollagen concentration was determined by colorimetric hydroxyproline assay using a procedure modified from Kivirikko *et al.* (1967) and Berg (1982) and assuming 10.11% hydroxyproline by weight for type I procollagen (Fiedler-Nagy *et al.*, 1981). The specific activity of the type I procollagen was typically 1500 $cpm \cdot \mu g^{-1}$. From 40, 175-cm^2 flasks of confluent cells, about 8 mg of purified type I procollagen was obtained from passage 7 cells and about 4 mg from passage 11 cells.

Preparation of pC-collagen—About 8 mg of purified type I procollagen was digested to completion by highly purified procollagen N-proteinase at 34 °C in 0.15 M NaCl, 5 mM $CaCl_2$, 0.05% Brij 35, 0.01% NaN_3, 50 mM Tris-HCl buffer, pH 7.4, at 20 °C. The procollagen N-proteinase was purified 5000-fold from chick embryo leg tendons[2] by a method based on that described by Tuderman *et al.* (1978) and Tuderman and Prockop (1982). Procollagen was digested exhaustively to ensure that uncleaved procollagen did not contaminate the pC-collagen preparation. Polyacrylamide gel electrophoresis of the samples demonstrated that no cleavage of the C-propeptides occurred. The reaction was stopped by the addition of 0.1 volume of 250 mM EDTA, 0.2% NaN_3, 50 mM Tris-HCl buffer, pH 7.4, at 20 °C, and the solution was placed on ice. The resulting pC-collagen was purified by Sephacryl S-300 (Pharmacia Biotechnology, Inc.) gel filtration chromatography. The pC-collagen mixture was passed over the column in two applications of 6 ml each. The column (2.25 × 60 cm) was equilibrated with 0.4 M NaCl, 1 M urea, 0.01% NaN_3, 0.1 M Tris-HCl buffer, pH 7.4, at 20 °C and was eluted at 4 °C and at a flow rate of 20 $ml \cdot h^{-1}$. Five fractions containing most of the radioactivity and corresponding to the void volume of the column contained the pC-collagen. The void volume fractions from both runs of the column were pooled (70 ml) and concentrated by pressure ultrafiltration as described above. The pC-collagen was stored in storage buffer at −15 °C.

Measurement of pC-collagen Thermal Stability by Proteinase Digestion—pC-collagen (400 $\mu g \cdot ml^{-1}$) in storage buffer was diluted 4-fold with water. Forty-μl aliquots were preincubated for 5 min at 36, 38, 40, 42, or 44 °C and then treated for 2 min at the same temperature with 4 μl of 1 $mg \cdot ml^{-1}$ trypsin and 2.5 $mg \cdot ml^{-1}$ chymotrypsin (Bruckner and Prockop, 1981) in a buffer consisting of 0.15 M NaCl, 10 mM EDTA, 0.01% Brij 35, 50 mM Tris-HCl buffer, pH 7.4, at 20 °C. Digestion was stopped by transference of the sample to boiling water and simultaneous addition of 44 μl of boiling 10% SDS. The samples were heated at 100 °C for 3 min. An equal volume of 2 × sample buffer consisting of 10% glycerol, 2% SDS, 2% 2-mercaptoethanol, 0.001% bromphenol blue, 0.125 M Tris-HCl buffer, pH 6.8, at 20 °C was added, and the samples were analyzed by polyacrylamide gel electrophoresis in the presence of SDS using a 6% separating gel and a 4% stacking gel (Laemmli, 1970). The radioactivity was displayed by fluorography using 20% 2,5-diphenyloxazole in glacial acetic acid and exposing dried gels to pre-flashed Kodak XAR-5 film at −70 °C. The film was found to have a linear response in the optical density range 0.15 to 2.0 and was therefore pre-flashed to an optical density of greater than 0.15 prior to exposure to the gel. Fluorograms were scanned using an LKB Ultroscan XL laser densitometer.

Purification of Procollagen C-proteinase—The purification procedures were the same as those described previously (Hojima *et al.*, 1985). Briefly, leg tendons were removed from a total of about 400 dozen 17-day chick embryos and stored at −20 °C. Tendons from about 20 dozen embryos were separately cultured in 200 ml of DMEM for 9 h at 37 °C. Medium was harvested, and the tendon tissues were further cultured twice for 12 h in DMEM. C-proteinase was purified from combined samples of medium by four successive chromatography steps: Green A Dye matrix gel, concanavalin A-Sepharose, heparin-Sepharose, and finally Sephacryl S-300 gel filtration chromatography. The purified enzyme preparation was concentrated by pressure ultrafiltration using a membrane (Amicon YM-30) with a 30,000-dalton molecular mass cut-off and dialyzed against fibril formation buffer (see below). The enzyme solution had an A_{280} of 0.116 and had 1,140 units $\cdot ml^{-1}$ of activity. One unit was defined as the amount of enzyme that cleaves 1 μg of type I procollagen in 1 h at 35 °C in the rapid assay (see below).

Assay of C-proteinase—To standardize the C-proteinase preparations, the rapid assay procedure (Hojima *et al.*, 1985) was used. The enzymic reaction was carried out in a final volume of 100 μl containing 12 $\mu g \cdot ml^{-1}$ chick type I ^{14}C-procollagen, varying amounts of diluted C-proteinase solution, and assay buffer consisting of 0.15 M NaCl, 0.01% NaN_3, 0.05% Brij 35, 5.5 mM $CaCl_2$, 50 mM Tris-HCl buffer, pH 7.4, at 20 °C. The reaction was carried out at 35 °C for 1 h and assayed by ethanol precipitation as described previously (Hojima *et al.*, 1985).

For the rapid assay, ^{14}C-labeled chick type I procollagen (Dehm and Prockop, 1972) was used as a substrate because of its high specific activity, typically 4×10^4 $cpm \cdot \mu g^{-1}$ procollagen. To prepare the procollagen, a suspension of matrix-free tendon cells was incubated with a mixture of ^{14}C-aminoacids (1 $\mu Ci \cdot ml^{-1}$) and the procollagen purified from the medium on a DEAE-cellulose column (Hoffmann *et al.*, 1976). The procollagen was concentrated to about 400 $\mu g \cdot ml^{-1}$ using pressure ultrafiltration as described above.

Gel Assay for pC-collagen Cleavage—To monitor the cleavage of pC-collagen under the conditions of fibril formation, mixtures (20 μl) of pC-collagen and C-proteinase were incubated in fibril formation buffer (see below) at the experimental temperature in 500-μl microcentrifuge tubes under an atmosphere of water-saturated 10% CO_2, 90% air. At times during incubation, a sample was transferred to an ice bath, an equal volume of 2 × sample buffer was added, and the solution was heated to 100 °C for 3 min. Samples were analyzed by polyacrylamide gel electrophoresis in the presence of SDS using 6% separating and 4% stacking polyacrylamide gels. Fluorograms were prepared and scanned using the methods described above.

Fibril Formation—pC-collagen substrate and C-proteinase were twice dialyzed against 600 volumes of fibril formation buffer that was a modified DMEM and consisted of 20 mM $NaHCO_3$, 117 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.81 mM $MgSO_4$, 1.03 mM NaH_2PO_4, 0.01% NaN_3, pH 7.3, at 20 °C. The buffer was stored at 4 °C under an atmosphere of 10% CO_2, and 90% air without precipitation or change in pH. Procollagen, pC-collagen, and C-proteinase were soluble at high concentrations in the buffer.

To initiate fibril formation, pC-collagen and C-proteinase solutions were mixed in a microcentrifuge tube at 4 °C, and the tube was briefly charged with water-saturated 10% CO_2, 90% air. The microcentrifuge tube was then quickly placed in a water bath set at a temperature in the range 21 to 41 °C.

For turbidity measurements, a 120-μl reaction mixture was preheated for 5 min in a water bath at the desired temperature, transferred to a preheated quartz microcuvette, and layered with an atmosphere of water-saturated 10% CO_2, 90% air. The 5-min incubation prior to turbidity measurements was used to prevent condensation and bubble formation in the cuvette and thereby to avoid the necessity of de-gassing solutions. The cuvette was sealed with a greased stopper. Changes in turbidity of the solution were assayed by absorbance at 313 nm in a Gilford spectrophotometer Model Response fitted with a temperature controlled cuvette holder. The cuvette chamber of the spectrophotometer was equilibrated with a constant flow of water-saturated 10% CO_2, 90% air. In experiments in which the temperature was varied, the concentration of C-proteinase was adjusted so that the rate of cleavage of pC-collagen was the same for each assay temperature. As noted previously (Hojima *et al.*, 1985), the activity of the enzyme increased 3.6-fold per 10 °C increase in assay temperature over the range 20 to 41 °C.

For assays of critical concentration, reaction mixtures of 40 μl were centrifuged at 13,000 × *g* for 4 min at room temperature in an Eppendorf centrifuge Model 5414. Small pellets were formed and the supernatants pipetted into a separate microcentrifuge tube. The supernatants were prepared for gel electrophoresis by adding 10 μl of 5 × sample buffer and heating at 100 °C for 3 min. The pellets were resuspended in 40 μl of fibril formation buffer and prepared for gel electrophoresis by adding 10 μl of 5 × sample buffer and heating at 100 °C for 3 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis using a 9% separating gel and a 4% stacking gel.

[2] Y. Hojima and D. J. Prockop, manuscript in preparation.

Fluorograms were prepared and scanned using the methods described above.

The concentration of collagen in the supernatant fraction was calculated by measuring the intensity of the α1(I) bands in fluorograms of the pellet and supernatant fractions and multiplying the relative amounts, corrected for molecular mass, by the initial concentration of pC-collagen. Because of the variability of the amounts of ^{14}C-labeled α1 chains, 3–5 exposures of each fluorogram were analyzed.

RESULTS

Preparation of the Type I pC-collagen Substrate—We elected to prepare type I pC-collagen by digesting purified type I procollagen with procollagen N-proteinase and purifying the resultant pC-collagen by gel filtration. Preliminary studies showed that preparations of chick procollagen isolated from the medium of cultured matrix-free tendon fibroblasts (Dehm and Prockop, 1972; Hoffmann *et al.*, 1976) were frequently contaminated with proteinases that slowly degraded native procollagen and rapidly degraded denatured procollagen. At first, the contaminating proteinase activity went unnoticed in dilute procollagen solutions and in procollagen solutions containing sodium chloride at concentrations greater than 0.2 M. In more concentrated solutions of procollagen and in low salt buffers, however, the proteinase activity was sufficiently high to interfere with fibril formation. The source of the proteinases was uncertain, but cell damage and lysosomal rupture could have occurred during preparation of the matrix-free cells. In contrast, it was found that preparations of type I procollagen from the medium of cultured human skin fibroblasts did not contain any detectable proteinase activity. Therefore, we prepared purified human procollagen from cultured fibroblasts, cleaved the procollagen to pC-collagen with N-proteinase, and purified the pC-collagen.

The pC-collagen was purified by Sephacryl S-300 gel filtration chromatography (Fig. 1*A*). The void volume fractions (22–26) contained the pC-collagen, and fractions 36–39 contained the cleaved N-propeptides. For fibril formation experiments, the pC-collagen was concentrated by pressure ultrafiltration on a membrane with a 10^5-dalton molecular mass cutoff. The trypsin-chymotrypsin test (Bruckner and Prockop, 1981) for triple helicity showed that the pC-collagen was native at 40–42 °C (not shown) and therefore had not suffered denaturation during pressure ultrafiltration. Also, incubation of the sample at 34 and 45 °C for 24 h showed that the preparation was free of any endogenous pC-collagen-degrading proteinase activities (Fig. 1*B*).

Collagen Fibril Formation by Cleavage of pC-collagen with C-proteinase—As a first step in the experiments described here, solubility properties of purified human procollagen and pC-collagen were examined. After concentration of the proteins on a membrane, large amounts of the protein appeared to be adherent to the membrane itself. Membranes were eluted in small volumes of the storage buffer (see above) previously shown to solubilize procollagen (Dehm and Prockop, 1972) better than standard physiological buffers. In repeated experiments and with repeated washing of membranes in minimal amounts of buffer, the highest concentrations of either procollagen or pC-collagen obtained was 0.5–1.0 mg·ml^{-1} at 4 °C in 0.4 M NaCl, 0.01% NaN_3, 0.1 M Tris-HCl buffer, pH 7.4, at room temperature. Therefore, the solubility limits of both proteins appear to be in this range.

The solutions of procollagen and pC-collagen were dialyzed at 4 °C against the physiological buffer used here for fibril formation and examined for evidence of aggregation or fibril formation. There were no changes in turbidity when the solutions in concentrations of up to 0.25 mg·ml^{-1} were warmed to 34 °C. Also, there was no evidence of fibril formation when the solutions were warmed to 34 °C, adsorbed onto electron microscopic grids, and the grids examined by negative staining electron microscopy. Fibrils or other aggregates were only seen with samples that contained trace amounts of collagen or pN-collagen.

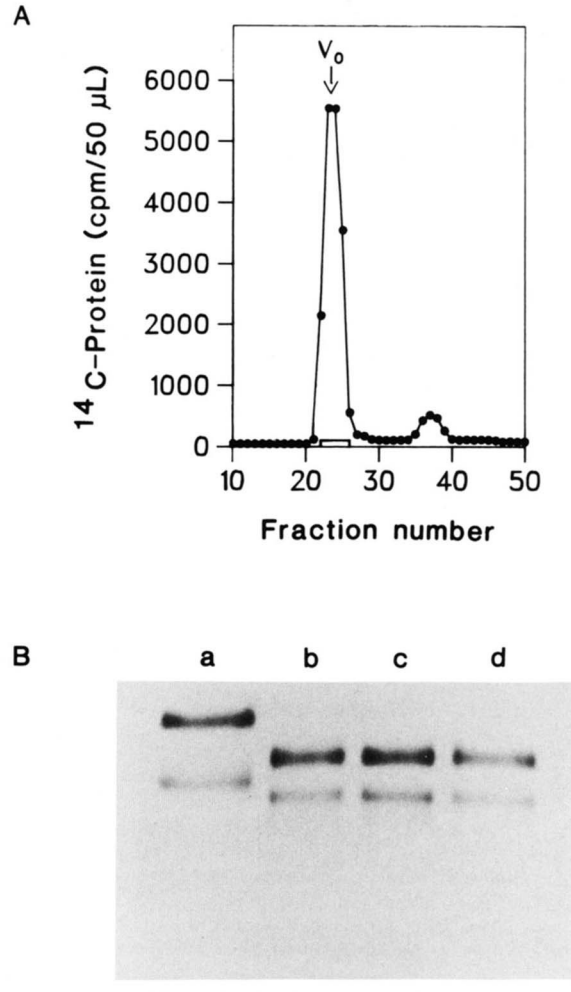

FIG. 1. **Preparation of type I pC-collagen.** *A*, Sephacryl S-300 gel filtration. Sample volume was 6 ml, column size 2.25 × 60 cm, and buffer 0.4 M NaCl, 1 M urea, 0.01% NaN_3, 0.1 M Tris-HCl buffer, pH 7.4, at 20 °C. The fraction volume was 5.4 ml, the flow rate was 20 ml·h^{-1}, and the temperature was 4 °C. *Bracketed fractions*, 22–26, were pooled. The void volume (V_0) was determined using blue dextran 2000 (Pharmacia Biotechnology, Inc.). *B*, heat test of pC-collagen. *a*, type I procollagen, no incubation; *b*, type I pC-collagen, no incubation; *c*, pC-collagen (0.4 mg·ml^{-1}) incubated at 34 °C for 24 h; *d*, pC-collagen (0.4 mg·ml^{-1}) incubated at 45 °C for 24 h. All samples were analyzed by gel electrophoresis and the radioactivity detected by fluorography.

Although pC-collagen itself did not form fibrils, cleavage of the pC-collagen to collagen with highly purified C-proteinase (Fig. 2*A*) produced a large increase in turbidity (Fig. 2*B*). In experiments in which a high concentration of 100 units·ml^{-1} C-proteinase were used with 0.25 mg·ml^{-1} pC-collagen, about 50% of the substrate was cleaved in 20 min and over 90% in 60 min (Fig. 2*B*). The increase in turbidity showed a lag period of about 20 min (Fig. 2*B*). Therefore, the kinetics seemed to follow the same pattern as the kinetics for fibril formation with extracted collagens (Williams *et al.*, 1978; Gelman *et al.*, 1979a, 1979b). The cleavage of pC-collagen by C-proteinase, however, generated a suspension of fibrils rather than a viscous gel that is formed by reconstituted fibrils. The fibrils formed here were like those described previously by Miyahara *et al.* (1982) after cleavage of pC-collagen by par-

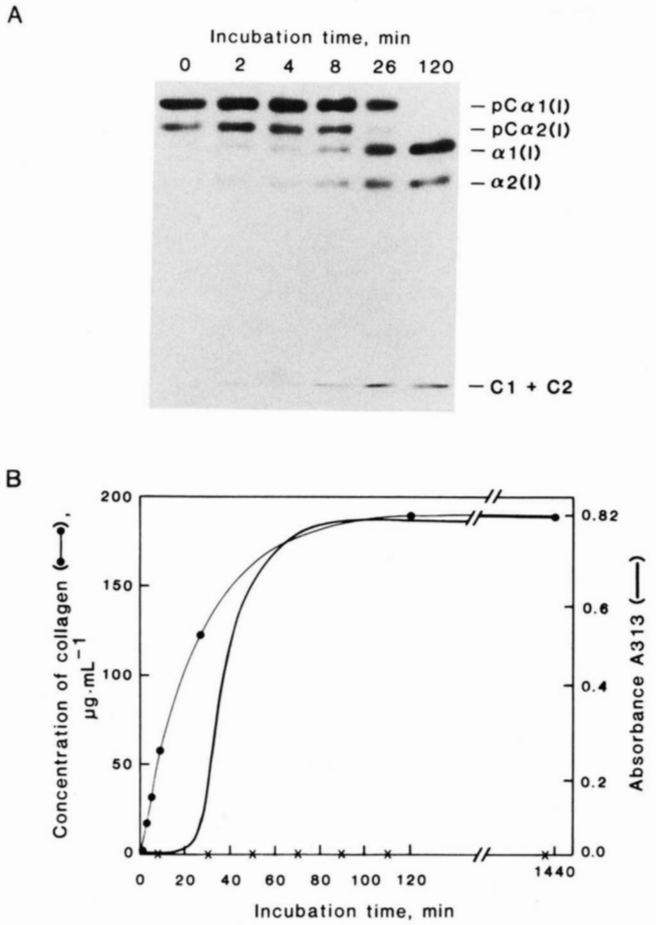

FIG. 2. **Formation of fibrils by cleavage of type I pC-collagen by procollagen C-proteinase.** *A*, analysis of reaction mixtures by gel electrophoresis. Six 40-μl mixtures of purified C-proteinase (100 units·ml^{-1}) and purified type I pC-collagen (250 μg·ml^{-1}) were incubated in fibril formation buffer at 34 °C. At each incubation time, the samples were prepared for electrophoresis as described under "Materials and Methods" and separated on a 6% polyacrylamide gel. pC-α1(I) chains were converted to α1(I) chains, and pC-α2(I) chains were converted to α2(I) chains by C-proteinase. C-propeptides from the pro-α1(I) and pro-α2(I) chains (*C1* and *C2*, respectively) were also generated in the reaction and co-migrated on the 6% separating gel. *B*, turbidometric assays. pC-collagen (250 μg·ml^{-1}) was incubated at 34 °C with or without C-proteinase (100 units·ml^{-1}). In the sample incubated with C-proteinase, the pC-collagen was cleaved (●——●) and the turbidity increased (——). About 50% of the substrate was cleaved before a turbidity was detected. Maximum turbidity occurred after more than 20 h after the beginning of the plateau phase and after complete cleavage of pC-collagen to collagen had occurred. In the sample incubated without C-proteinase, there was no cleavage of the pC-collagen or increase in turbidity (×——×).

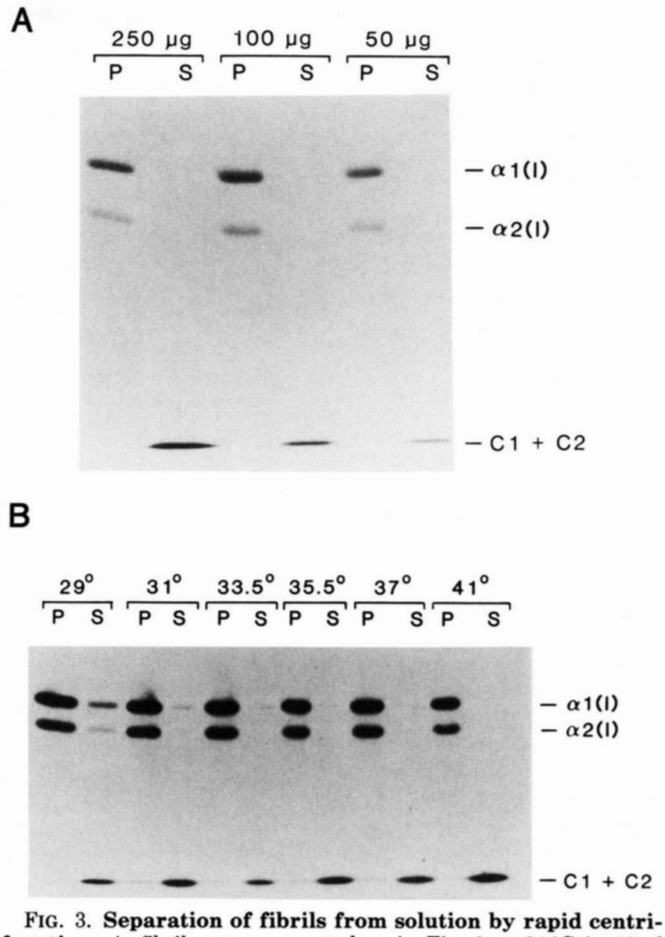

FIG. 3. **Separation of fibrils from solution by rapid centrifugation.** *A*, fibrils were generated as in Fig. 2 at 34 °C in 40-μl volumes, and the mixtures centrifuged at 13,000 × *g* for 4 min. Pellet and supernatant fractions were prepared for gel electrophoresis and separated on a 6% polyacrylamide reducing gel. The radioactivity was detected by fluorography. Pellet (*P*) and supernatant (*S*) fractions from reaction mixtures with (*a*) 250 μg·ml^{-1} pC-collagen, (*b*) 100 μg·ml^{-1} pC-collagen, and (*c*) 50 μg·ml^{-1} pC-collagen. All samples were incubated with 100 units·ml^{-1} C-proteinase. The pellets contained only α1(I) and α2(I) chains of collagen and the supernatants contained the cleaved C-propeptides plus trace amounts of α chains that were apparent only after overexposure of films (not shown). *B*, effect of temperature on the critical concentration of collagen. pC-collagen (100 μg·ml^{-1}) and C-proteinase were incubated for 24 h in fibril formation buffer at different temperatures. The concentration of C-proteinase was varied from 42 to 185 units·ml^{-1} so that the rate of cleavage remained constant at the different temperatures. Samples were centrifuged at 13,000 × *g* for 4 min. Pellet and supernatant fractions were prepared for gel electrophoresis and the samples separated on a 9% reducing gel. Pellet fractions contained α1(I) and α2(I) chains of collagen only, and the supernatants contained the cleaved C-propeptides and soluble collagen.

tially purified C-proteinase. They were visible to the eye, large in diameter, and needle-shaped. The collagen fibrils formed were dissociated by lowering the temperature of the solution to 10 °C. Increasing the temperature to 34 °C reconstituted the fibrils and demonstrated the complete reversibility of the assembly process (not shown). Most importantly for the analyses developed below, fibrils of essentially the same appearance were formed when the reaction was carried out at temperatures ranging from as low as 29 °C to as high as 41 °C.

Separation of Fibrils from Solution by Centrifugation—Although the fibrils formed were large and filled the cuvette (Miyahara *et al.*, 1982), we found it easy to separate the fibrils by centrifugation at 13,000 × *g* for 4 min. The fibrils were recovered in a small pellet. As indicated in Fig. 3, only α chains of collagen were recovered in the pellet after the pC-collagen was fully cleaved by C-proteinase at 34 °C and fibrils had formed at 34 °C for 10 h or more. The C-propeptides were found in the supernatant of the fibrils. Scanning of fluorograms indicated that less than 0.1% of the labeled C-propeptides were recovered in the pellet of fibrils. Similar results were obtained at 29 and 41 °C (see below). Therefore, it was apparent that the C-propeptides were not an integral part of the fibrils formed.

Critical Concentration of Collagen—Overexposure of fluorograms such as the ones shown in Fig. 3 indicated that a small amount of collagen was present in the supernatant of centrifuged fibril preparations. As indicated in Fig. 4, the amount of collagen in the supernatant reached an equilibrium value after 10–24 h. The time for reaching equilibrium de-

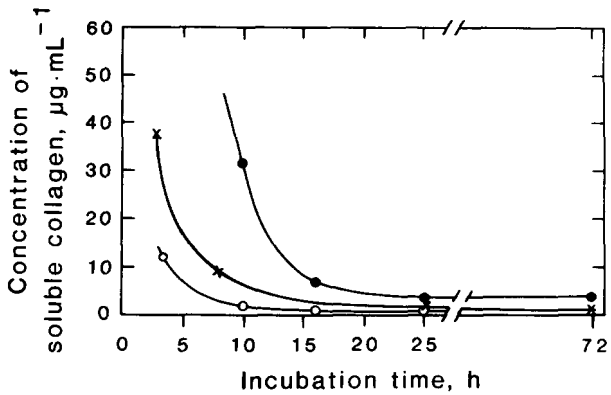

FIG. 4. **Determination of the equilibrium point for critical concentration measurements.** pC-Collagen (100 $\mu g \cdot ml^{-1}$) and C-proteinase (100 units $\cdot ml^{-1}$) were incubated at 29 °C (●——●), 33 °C (×——×), and at 35 °C (○——○). At times indicated, samples were centrifuged at 13,000 × *g* for 4 min and the pellet and supernatant fractions prepared for gel electrophoresis. Fluorograms were scanned using a laser densitometer and the concentration of soluble collagen determined at each temperature.

TABLE I

Effect of substrate concentration and enzymic cleavage rate on critical concentration at 34 °C and the effect of temperature on critical concentration

Temperature	Initial pC-collagen concentration	Enzyme activity	Mean critical concentration of collagen	*n*
°C	$\mu g \cdot ml^{-1}$	$units \cdot ml^{-1}$	$\mu g \cdot ml^{-1}$ ± S.E.	
29	100	100	4.73 ± 0.84	3
31	100	100	1.56 ± 0.52	3
33.5	100	100	0.78 ± 0.22	3
34	250	5	0.83 ± 0.37	3
	250	50	0.78 ± 0.18	3
	250	100	0.80 ± 0.19	3
35.5	100	100	0.55 ± 0.12	3
37	100	100	0.42 ± 0.10	4
	150	60		
38	100	100	0.34 ± 0.13	3
41	100	100	0.12 ± 0.06	3

creased between 29 and 35 °C (Fig. 4) and increased again between 35 and 41 °C (not shown) but never exceeded about 24 h. The value for collagen in solution and in equilibrium with fibrils is the critical concentration for polymerization as defined for other self-assembly systems (see Oosawa and Asakura, 1975).

As expected, varying the initial pC-collagen concentration over a 2.5-fold range did not change the critical concentration of collagen (Table I). Also, varying enzyme concentrations over a 20-fold range at 34 °C had no effect. In addition, the same value at 34 °C was obtained after the samples were equilibrated at 29 °C for 24 h, and then the temperature jumped to 34 °C for 10 h. Therefore, the results were consistent with the conclusion that the final concentration of collagen in solution was an equilibrium value and that the system behaved as a phase transition phenomenon (Gerber *et al.*, 1973; Lauffer, 1975; Oosawa and Asakura, 1975).

As expected, varying the temperature had a major effect on the critical concentration (Figs. 3*B* and 5). As discussed elsewhere (see Lauffer, 1975 and Oosawa and Asakura, 1975), the critical concentration (c_c) can be substituted for the reciprocal of the equilibrium constant for monomer addition to fibrils. Therefore, it can be used in a derivation of the van't Hoff equation to calculate both the enthalpy change (ΔH) and the entropy change (ΔS) for polymerization according to the formula:

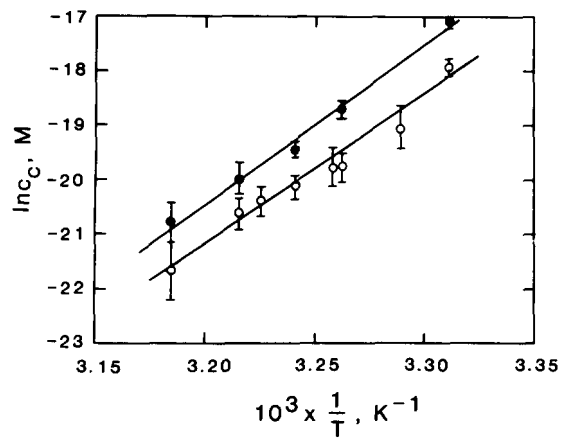

FIG. 5. **Determination of the thermodynamic parameters of collagen fibril formation *de novo*.** Critical concentration data were plotted on a van't Hoff graph as natural logarithm of the critical concentration (M) against inverse of absolute temperature (K^{-1}). Mean values at each temperature were fitted to a straight line by least-squares linear regression. The slope of the line equals $\Delta H/R$ where R is the molar gas constant and the intercept on the ln c_c axis when $1/T$ is zero equals ΔS. For fibroblast culture GM 3349 (○——○), ΔH = +53 kcal $\cdot$ mol^{-1}, ΔS = +210 cal $\cdot K^{-1} \cdot$ mol^{-1} (r = 0.98, n = 8) and fibroblast culture JIMM-86 (●——●), ΔH = +58 kcal $\cdot$ mol^{-1}, ΔS = +230 cal $\cdot K^{-1} \cdot$ mol^{-1} (r = 0.99, n = 5).

TABLE II

Thermodynamic parameters for systems of protein self-assembly

Protein	ΔG^a	ΔH	ΔS	Reference
	kcal $\cdot$ mol^{-1}		cal $\cdot K^{-1} \cdot$ mol^{-1}	
G-actin		+10–15		Asakura *et al.* (1960)
G-ADP-actin		+10–15		Kasai (1969)
Flagella	−1.9[b]	+101	+332	Gerber *et al.* (1973)
Tobacco mosaic virus protein	−10.0[b]	+34	+139	Paglini and Lauffer (1968)
Sickle cell Hb	−11.4[b]	+68	+256	Murayama (1972)
Type I collagen				
Extracted	−23			Cooper (1970)
De novo[c]	−13	+56	+220	

[a] At 37 °C.
[b] Values calculated here from published data.
[c] Mean of two values obtained here.

$$c_c \cdot K_{eq} = 1$$

$$\ln c_c = -\ln K_{eq} = \frac{\Delta H}{RT} - \frac{\Delta S}{R}$$

where K_{eq} is the thermodynamic equilibrium constant, R is the molar gas constant, and T is the absolute temperature.

As shown in Fig. 5, the plot of ln c_c *versus* inverse of absolute temperature gave a straight line, an observation consistent with a simple thermodynamic process. The values for ΔH and ΔS calculated from the plot were +53 kcal $\cdot$ mol^{-1} and +210 cal $\cdot K^{-1} \cdot$ mol^{-1}, respectively (Table II). The Gibbs energy change was −12 kcal $\cdot$ mol^{-1} at 37 °C. To show that the values obtained for the thermodynamic parameters of assembly were not strictly dependent on the fibroblast culture, another normal fibroblast culture, JIMM-86, was examined. The van't Hoff plot for fibril assembly from collagen derived from JIMM-86 gave values for ΔH and ΔS of +58 kcal $\cdot$ mol^{-1} and +230 cal $\cdot K^{-1} \cdot$ mol^{-1}, respectively. The Gibbs energy change was −13 kcal $\cdot$ mol^{-1}. The values indicate that assembly of collagen fibrils *de novo* is endothermic and entropy-driven.

DISCUSSION

A number of attempts were made in the past to measure the thermodynamic parameters of collagen fibril formation using extracted collagens (Cooper, 1970; Williams *et al.*, 1978; Na, 1986a). Among the problems encountered was the fact that fibril assembly occurs very poorly in physiological buffers with extracted collagens, particularly at physiological temperatures. Also, using extracted collagens, it is difficult to measure the low concentrations of collagen monomer in equilibrium with fibrils. For example, Cooper (1970) measured the solubility of collagen extracted from calf skin over the temperature range of 20 to 37 °C. His data indicated a Gibbs energy change of -23 kcal·mol^{-1}, a value about twice that observed here. However, he employed a phosphate concentration that was 100 mM, or about 100-fold higher than the physiological concentration, to avoid gels that were "weak and almost water-clear." Also, he encountered an unexplained upward curvature of the solubility curve as the temperature was raised from 34 to 37 °C. Williams *et al.* (1978) determined that the solubility of collagen in 30 mM phosphate was less than 7 μg·ml^{-1} but were unable to assay it below this concentration. More recently, Na (1986a) estimated that the critical concentration of extracted collagen in 30 mM phosphate was less than 10 μg·ml^{-1} but, again, was unable to assay the concentration directly.

The system used here to generate collagen fibrils *de novo* has several important features. The pC-collagen used as a substrate was obtained after cleavage of purified procollagen with the N-proteinase shown to be involved in the normal processing of procollagen (Tuderman *et al.*, 1978; Tuderman and Prockop, 1982). The pC-collagen was repurified and then used to generate collagen in a controlled manner by cleavage with a highly purified preparation of the C-proteinase that was shown to cleave the molecule at the same site that is cleaved *in vivo* (Hojima *et al.*, 1985). Using this system, we were able to assay directly, for the first time, the critical concentration of collagen in equilibrium with collagen fibrils in a physiological buffer and over a physiological range of temperatures. Furthermore, the critical concentration was shown to be a true equilibrium value for the system as observed in classical crystallization, condensation, and phase transition phenomena.

As noted by others (Oosawa and Asakura, 1975; Lauffer, 1975), the critical concentration can be substituted for the reciprocal of the equilibrium constant for monomer addition. Therefore, by using a derivation of the van't Hoff equation, the thermodynamic parameters of collagen fibril assembly were derived for the temperature range 29 to 41 °C. As expected for a simple polymerization system, the van't Hoff relationship was linear over this temperature range. In addition, the results indicated that the process was endothermic and entropy-driven with thermodynamic parameters similar to those for other well studied biological systems of polymerization (Table II). Therefore, polymerization of collagen is similar to that of tobacco mosaic virus protein (Stevens and Lauffer, 1965; Banerjee and Lauffer, 1966; Paglini and Lauffer, 1968), action (Asakura *et al.*, 1960; Kasai, 1969), and flagella (Gerber *et al.*, 1973) in the sense that the major driving force is the increase in entropy associated with the loss of water bound to the monomer as the polymerization occurs.

Under the conditions employed here, the pC-collagen used as a substrate did not form any apparent aggregates in concentrations of up to 0.25 mg·ml^{-1} at 34 °C. Also, after cleavage of the pC-collagen to collagen, less than 0.1% of the C-propeptide was found in the fibrillar phase. Therefore, neither the initial pC-collagen nor the C-propeptides are an integral part of the fibrils formed. However, the results do not exclude the possibility that the N- and C-propeptides may help generate intermediates in fibril assembly or that pN-collagen or pC-collagen may participate in assembly or disassembly of intermediates (Lapiere and Nusgens, 1974; Trelstad and Hayashi, 1979; Bruns *et al.*, 1979; Hulmes *et al.*, 1983; Hulmes, 1983; Fleischmajer *et al.*, 1983, 1986). We are currently exploring these possibilities and the effects of mutations that alter the primary structure of type I procollagen (for review, see Prockop and Kivirikko, 1984).

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